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(54) Title: RECOMBINANT VIRUS IMMUNOTHERAPY

(57) Abstract

Attenuated recombinant viruses containing DNA coding for a cytokine and/or a tumor associated antigen, as well as methods and compositions employing the viruses, are disclosed and claimed. The recombinant viruses can be NYVAC or ALVAC recombinant viruses. The DNA can code for at least one of: human tumor necrosis factor; nuclear phosphoprotein p53, wildtype or mutant; human melanoma-associated antigen; IL-2; IFN γ ; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen. The recombinant viruses and gene products therefrom are useful for cancer therapy.

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RECOMBINANT VIRUS IMMUNOTHERAPY

FIELD OF THE INVENTION

The present invention relates to a modified poxvirus and to methods of making and using the same. More in particular, the invention relates to improved vectors for the insertion and expression of foreign genes for use as safe immunization vehicles to protect against a variety of pathogens, as well as for use in immunotherapy.

Several publications are referenced in this application. Full citation to these references is found at the end of the specification immediately preceding the claims or where the publication is mentioned; and each of these publications is hereby incorporated herein by reference. These publications relate to the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other poxviruses have been used for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious poxvirus involves recombination between pox DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus (Piccini et al., 1987).

Specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described in U.S. Patent Nos. 4,769,330, 4,772,848, 4,603,112, 5,100,587, and 5,179,993, the disclosures of which are incorporated herein by reference.

First, the DNA gene sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene

linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid 5 construct is then amplified by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1982).

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell 10 culture, e.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences. The term 15 "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

Genetic recombination is in general the exchange of 20 homologous sections of DNA between two strands of DNA. In certain viruses RNA may replace DNA. Homologous sections of nucleic acid are sections of nucleic acid (DNA or RNA) which have the same sequence of nucleotide bases.

25 Genetic recombination may take place naturally during the replication or manufacture of new viral genomes within the infected host cell. Thus, genetic recombination between viral genes may occur during the viral replication cycle that takes place in a host cell 30 which is co-infected with two or more different viruses or other genetic constructs. A section of DNA from a first genome is used interchangeably in constructing the section of the genome of a second co-infecting virus in which the DNA is homologous with that of the first viral 35 genome.

However, recombination can also take place between sections of DNA in different genomes that are not

perfectly homologous. If one such section is from a first genome homologous with a section of another genome except for the presence within the first section of, for example, a genetic marker or a gene coding for an antigenic determinant inserted into a portion of the homologous DNA, recombination can still take place and the products of that recombination are then detectable by the presence of that genetic marker or gene in the recombinant viral genome. Additional strategies have recently been reported for generating recombinant vaccinia virus (Scheiflinger et al., 1992; Merchlinsky and Moss, 1992).

Successful expression of the inserted DNA genetic sequence by the modified infectious virus requires two conditions. First, the insertion must be into a nonessential region of the virus in order that the modified virus remain viable. The second condition for expression of inserted DNA is the presence of a promoter in the proper relationship to the inserted DNA. The promoter must be placed so that it is located upstream from the DNA sequence to be expressed.

Vaccinia virus has been used successfully to immunize against smallpox, culminating in the worldwide eradication of smallpox in 1980. In the course of its history, many strains of vaccinia have arisen. These different strains demonstrate varying immunogenicity and are implicated to varying degrees with potential complications, the most serious of which are post-vaccinial encephalitis and generalized vaccinia (Behbehani, 1983).

With the eradication of smallpox, a new role for vaccinia became important, that of a genetically engineered vector for the expression of foreign genes. Genes encoding a vast number of heterologous antigens have been expressed in vaccinia, often resulting in protective immunity against challenge by the corresponding pathogen (reviewed in Tartaglia et al.,

1990a,b).

The genetic background of the vaccinia vector has been shown to affect the protective efficacy of the expressed foreign immunogen. For example, expression of 5 Epstein Barr Virus (EBV) gp340 in the Wyeth vaccine strain of vaccinia virus did not protect cottontop tamarins against EBV virus induced lymphoma, while expression of the same gene in the WR laboratory strain of vaccinia virus was protective (Morgan et al., 1988).

10 A fine balance between the efficacy and the safety of a vaccinia virus-based recombinant vaccine candidate is extremely important. The recombinant virus must present the immunogen(s) in a manner that elicits a protective immune response in the vaccinated animal but 15 lacks any significant pathogenic properties. Therefore attenuation of the vector strain would be a highly desirable advance over the current state of technology.

20 A number of vaccinia genes have been identified which are non-essential for growth of the virus in tissue culture and whose deletion or inactivation reduces virulence in a variety of animal systems.

25 The gene encoding the vaccinia virus thymidine kinase (TK) has been mapped (Hruby et al., 1982) and sequenced (Hruby et al., 1983; Weir et al., 1983). Inactivation or complete deletion of the thymidine kinase gene does not prevent growth of vaccinia virus in a wide variety of cells in tissue culture. TK⁻ vaccinia virus is also capable of replication *in vivo* at the site of inoculation in a variety of hosts by a variety of routes.

30 It has been shown for herpes simplex virus type 2 that intravaginal inoculation of guinea pigs with TK⁻ virus resulted in significantly lower virus titers in the spinal cord than did inoculation with TK⁺ virus (Stanberry et al., 1985). It has been demonstrated that 35 herpesvirus encoded TK activity *in vitro* was not important for virus growth in actively metabolizing cells, but was required for virus growth in quiescent

cells (Jamieson et al., 1974).

Attenuation of TK⁻ vaccinia has been shown in mice inoculated by the intracerebral and intraperitoneal routes (Buller et al., 1985). Attenuation was observed 5 both for the WR neurovirulent laboratory strain and for the Wyeth vaccine strain. In mice inoculated by the intradermal route, TK⁻ recombinant vaccinia generated equivalent anti-vaccinia neutralizing antibodies as compared with the parental TK⁺ vaccinia virus, indicating 10 that in this test system the loss of TK function does not significantly decrease immunogenicity of the vaccinia virus vector. Following intranasal inoculation of mice with TK⁻ and TK⁺ recombinant vaccinia virus (WR strain), significantly less dissemination of virus to other 15 locations, including the brain, has been found (Taylor et al., 1991a).

Another enzyme involved with nucleotide metabolism is ribonucleotide reductase. Loss of virally encoded ribonucleotide reductase activity in herpes simplex virus 20 (HSV) by deletion of the gene encoding the large subunit was shown to have no effect on viral growth and DNA synthesis in dividing cells *in vitro*, but severely compromised the ability of the virus to grow on serum starved cells (Goldstein et al., 1988). Using a mouse 25 model for acute HSV infection of the eye and reactivatable latent infection in the trigeminal ganglia, reduced virulence was demonstrated for HSV deleted of the large subunit of ribonucleotide reductase, compared to the virulence exhibited by wild type HSV (Jacobson et 30 al., 1989).

Both the small (Slabaugh et al., 1988) and large (Schmitt et al., 1988) subunits of ribonucleotide reductase have been identified in vaccinia virus. Insertional inactivation of the large subunit of 35 ribonucleotide reductase in the WR strain of vaccinia virus leads to attenuation of the virus as measured by intracranial inoculation of mice (Child et al., 1990).

The vaccinia virus hemagglutinin gene (HA) has been mapped and sequenced (Shida, 1986). The HA gene of vaccinia virus is nonessential for growth in tissue culture (Ichihashi et al., 1971). Inactivation of the HA gene of vaccinia virus results in reduced neurovirulence in rabbits inoculated by the intracranial route and smaller lesions in rabbits at the site of intradermal inoculation (Shida et al., 1988). The HA locus was used for the insertion of foreign genes in the WR strain (Shida et al., 1987), derivatives of the Lister strain (Shida et al., 1988) and the Copenhagen strain (Guo et al., 1989) of vaccinia virus. Recombinant HA⁻ vaccinia virus expressing foreign genes have been shown to be immunogenic (Guo et al., 1989; Itamura et al., 1990; Shida et al., 1988; Shida et al., 1987) and protective against challenge by the relevant pathogen (Guo et al., 1989; Shida et al., 1987).

Cowpox virus (Brighton red strain) produces red (hemorrhagic) pocks on the chorioallantoic membrane of chicken eggs. Spontaneous deletions within the cowpox genome generate mutants which produce white pocks (Pickup et al., 1984). The hemorrhagic function (u) maps to a 38 kDa protein encoded by an early gene (Pickup et al., 1986). This gene, which has homology to serine protease inhibitors, has been shown to inhibit the host inflammatory response to cowpox virus (Palumbo et al., 1989) and is an inhibitor of blood coagulation.

The u gene is present in WR strain of vaccinia virus (Kotwal et al., 1989b). Mice inoculated with a WR vaccinia virus recombinant in which the u region has been inactivated by insertion of a foreign gene produce higher antibody levels to the foreign gene product compared to mice inoculated with a similar recombinant vaccinia virus in which the u gene is intact (Zhou et al., 1990). The u region is present in a defective nonfunctional form in Copenhagen strain of vaccinia virus (open reading frames B13 and B14 by the terminology reported in Goebel et al.,

1990a,b).

Cowpox virus is localized in infected cells in cytoplasmic A type inclusion bodies (ATI) (Kato et al., 1959). The function of ATI is thought to be the protection of cowpox virus virions during dissemination from animal to animal (Bergoin et al., 1971). The ATI region of the cowpox genome encodes a 160 kDa protein which forms the matrix of the ATI bodies (Funahashi et al., 1988; Patel et al., 1987). Vaccinia virus, though containing a homologous region in its genome, generally does not produce ATI. In WR strain of vaccinia, the ATI region of the genome is translated as a 94 kDa protein (Patel et al., 1988). In Copenhagen strain of vaccinia virus, most of the DNA sequences corresponding to the ATI region are deleted, with the remaining 3' end of the region fused with sequences upstream from the ATI region to form open reading frame (ORF) A26L (Goebel et al., 1990a,b).

A variety of spontaneous (Altenburger et al., 1989; Drillien et al., 1981; Lai et al., 1989; Moss et al., 1981; Paez et al., 1985; Panicali et al., 1981) and engineered (Perkus et al., 1991; Perkus et al., 1989; Perkus et al., 1986) deletions have been reported near the left end of the vaccinia virus genome. A WR strain 25 of vaccinia virus with a 10 kb spontaneous deletion (Moss et al., 1981; Panicali et al., 1981) was shown to be attenuated by intracranial inoculation in mice (Buller et al., 1985). This deletion was later shown to include 17 potential ORFs (Kotwal et al., 1988b). Specific genes 30 within the deleted region include the virokine N1L and a 35 kDa protein (C3L, by the terminology reported in Goebel et al., 1990a,b). Insertional inactivation of N1L reduces virulence by intracranial inoculation for both normal and nude mice (Kotwal et al., 1989a). The 35 kDa 35 protein is secreted like N1L into the medium of vaccinia virus infected cells. The protein contains homology to the family of complement control proteins, particularly

the complement 4B binding protein (C4bp) (Kotwal et al., 1988a). Like the cellular C4bp, the vaccinia 35 kDa protein binds the fourth component of complement and inhibits the classical complement cascade (Kotwal et al., 1990). Thus the vaccinia 35 kDa protein appears to be involved in aiding the virus in evading host defense mechanisms.

The left end of the vaccinia genome includes two genes which have been identified as host range genes, K1L (Gillard et al., 1986) and C7L (Perkus et al., 1990). Deletion of both of these genes reduces the ability of vaccinia virus to grow on a variety of human cell lines (Perkus et al., 1990).

Two additional vaccine vector systems involve the use of naturally host-restricted poxviruses, avipoxviruses. Both fowlpoxvirus (FPV) and canarypoxvirus (CPV) have been engineered to express foreign gene products. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. The virus causes an economically important disease of poultry which has been well controlled since the 1920's by the use of live attenuated vaccines. Replication of the avipox viruses is limited to avian species (Matthews, 1982b) and there are no reports in the literature of avipoxvirus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipoxvirus based vaccine vectors in veterinary and human applications an attractive proposition.

FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant (Taylor et al., 1988a). After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a

heterologous virulent influenza virus challenge (Taylor et al., 1988a). FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor et al., 1990; Edbauer et al., 1990).

5 Despite the host-restriction for replication of FPV and CPV to avian systems, recombinants derived from these viruses were found to express extrinsic proteins in cells of nonavian origin. Further, such recombinant viruses were shown to elicit immunological responses directed 10 towards the foreign gene product and where appropriate were shown to afford protection from challenge against the corresponding pathogen (Tartaglia et al., 1993 a,b; Taylor et al., 1992; 1991b; 1988b).

In the past, viruses have been shown to have utility 15 in cancer immunotherapy, in that, they provide a means of enhancing tumor immunoresponsiveness. Examples exist showing that viruses such as Newcastle disease virus (Cassel et al., 1983), influenza virus (Lindenmann, 1974; Lindenmann, 1967), and vaccinia virus (Wallack et al., 20 1986; Shimizu et al., 1988; Shimizu et al. 1984; Fujiwara et al., 1984) may act as tumor-modifying antigens or adjuvants resulting in inducing tumor-specific and tumor-nonspecific immune effector mechanisms. Due to advances 25 in the fields of immunology, tumor biology, and molecular biology, however, such approaches have yielded to more directed immunotherapeutic approaches for cancer.

Genetic modification of tumor cells and immune effector cells (i.e. tumor-infiltrating lymphocytes; TILs) to express, for instance cytokines, have provided 30 encouraging results in animal models and humans with respect to augmenting tumor-directed immune responses (Pardoll, 1992; Rosenberg, 1992). Further, the definition of tumor-associated antigens (TAAs) has provided the opportunity to investigate their role in the 35 immunobiology of certain cancers which may eventually be applied to their use in cancer prevention or therapy (van der Bruggen, 1992).

Advances in the use of eukaryotic vaccine vectors have provided a renewed interest in viruses in cancer prevention and therapy. Among the viruses engineered to express foreign gene products are adenoviruses, adeno-
5 associated virus, baculovirus, herpesviruses, poxviruses, and retroviruses. Most notably, retrovirus-, adenovirus-, and poxvirus-based recombinant viruses have been developed with the intent of *in vivo* utilization in the areas of vector-based vaccines, gene therapy, and cancer therapy (Tartaglia, *in press*; Tartaglia, 1990).

10 Immunotherapeutic approaches to combat cancers or neoplasia can take the form of classical vaccination schemes or cell-based therapies. Immunotherapeutic vaccination is the concept of inducing or enhancing 15 immune responses of the cancer patient to antigenic determinants that are uniquely expressed or expressed at increased levels on tumor cells. Tumor-associated antigens (TAAs) are usually of such weak immunogenicity as to allow progression of the tumor unhindered by the 20 patient's immune system. Under normal circumstances, the severity of the disease-state associated with the tumor progresses more rapidly than the elaboration of immune responses, if any, to the tumor cells. Consequently, the patient may succumb to the neoplasia before a sufficient 25 immune response is mounted to control and prevent growth and spread of the tumor.

Poxvirus vector technology has been utilized to elicit immunological responses to TAAs. Examples exist demonstrating the effectiveness of poxvirus-based 30 recombinant viruses expressing TAAs in animal models in the immunoprophylaxis and immunotherapy of experimentally-induced tumors. The gene encoding carcinembryonic antigen (CEA) was isolated from human colon tumor cells and inserted into the vaccinia virus 35 genome (Kaufman et al., 1991). Inoculation of the vaccinia-based CEA recombinant elicited CEA-specific antibodies and an antitumor effect in a murine mouse

model. This recombinant virus has been shown to elicit humoral and cell-mediated responses in rhesus macaques (Kantor et al., 1993). The human melanoma TAA, p97, has also been inserted into vaccinia virus and shown to 5 protect mice from tumor transplants (Hu et al., 1988; Estin et al., 1988). A further example was described by Bernards et al. (1987). These investigators constructed a vaccinia recombinant that expressed the extracellular domain of the rat neu-encoded transmembrane glycoprotein, 10 p185. Mice immunized with this recombinant virus developed a strong humoral response against the neu gene product and were protected against subsequent tumor challenge. Vaccinia virus recombinants expressing either a secreted or membrane-anchored form of a breast cancer- 15 associated epithelial tumor antigen (ETA) have been generated for evaluation in the active immunotherapy of breast cancer (Hareveni et al., 1991; 1990). These recombinant viruses have been shown to elicit anti-ETA antibodies in mice and to protect mice against a 20 tumorigenic challenge with a ras-transformed Fischer rat fibroblast line expressing either form of ETA (Hareveni et al., 1990). Further, vaccinia virus recombinants expressing the polyoma virus-derived T-Ag were shown efficacious for prevention and therapy in a mouse tumor 25 model system (Lathe et al., 1987).

Recombinant vaccinia viruses have also been used to express cytokine genes (Reviewed by Ruby et al., 1992). Expression of certain cytokines (IL-2, IFN- α , TNF- α) lead to self-limiting vaccinia virus infection in mice and, in 30 essence, act to attenuate the virus. Expression of other cytokines (i.e. IL-5, IL-6) were found to modulate the immune response to co-expressed extrinsic immunogens (Reviewed by Ruby et al., 1992).

Frequently, immune responses against tumor cells are 35 mediated by T cells, particularly cytotoxic T lymphocytes (CTLs); white blood cells capable of killing tumor cells and virus-infected cells (Greenberg, 1991). The behavior

of CTLs is regulated by soluble factors termed cytokines. Cytokines direct the growth, differentiation, and functional properties of CTLs, as well as, other immune effector cells.

5 Cell-based immunotherapy has been shown to provide effective therapy for viruses and tumors in animal models (Greenberg, 1991; Pardoll, 1992; Riddel et al., 1992). Cytomegalovirus (CMV)-specific CTL clones from bone marrow donors have recently been isolated. These clones 10 were propagated and expanded *in vitro* and ultimately returned to immunodeficient bone marrow patients. These transferred CMV-specific CTL clones provided no toxic-effects and provided persistent reconstitution of CD8⁺ CMV-specific CTL responses preventing CMV infection in 15 the transplant patient (Riddel et al., 1992).

There exists two forms of cell-based immunotherapy. These are adoptive immunotherapy, which involves the expansion of tumor reactive lymphocytes *in vitro* and reinfusion into the host, and active immunotherapy, which 20 involves immunization of tumor cells to potentially enhance existing or to elicit novel tumor-specific immune responses and provide systemic anti-tumor immunity. Immunotherapeutic vaccination is the concept of inducing or enhancing immune responses of the cancer patient to 25 antigenic determinants that are uniquely expressed or expressed at increased levels on tumor cells.

It can be appreciated that provision of novel strains, such as NYVAC, ALVAC, and TROVAC having enhanced safety would be a highly desirable advance over the 30 current state of technology. For instance, so as to provide safer vaccines or safer products from the expression of a gene or genes by a virus.

OBJECTS OF THE INVENTION

It is therefore an object of this invention to 35 provide modified recombinant viruses, which viruses have enhanced safety, and to provide a method of making such recombinant viruses.

It is an additional object of this invention to provide a recombinant poxvirus vaccine having an increased level of safety compared to known recombinant poxvirus vaccines.

5 It is a further object of this invention to provide a modified vector for expressing a gene product in a host, wherein the vector is modified so that it has attenuated virulence in the host.

10 It is another object of this invention to provide a method for expressing a gene product in a cell cultured in vitro using a modified recombinant virus or modified vector having an increased level of safety.

15 These and other objects and advantages of the present invention will become more readily apparent after consideration of the following.

STATEMENT OF THE INVENTION

In one aspect, the present invention relates to a modified recombinant virus having inactivated virus-encoded genetic functions so that the recombinant virus 20 has attenuated virulence and enhanced safety. The functions can be non-essential, or associated with virulence. The virus is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus. The modified 25 recombinant virus can include, within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein, e.g., derived from a pathogen, a tumor associated antigen, a cytokine, or combination thereof.

30 In another aspect, the present invention relates to a vaccine for inducing an antigenic response in a host animal inoculated with the vaccine, said vaccine including a carrier and a modified recombinant virus having inactivated nonessential virus-encoded genetic 35 functions so that the recombinant virus has attenuated virulence and enhanced safety. The virus used in the vaccine according to the present invention is

advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus. The modified recombinant virus can include, within a non-essential region of the virus genome, a 5 heterologous DNA sequence which encodes an antigenic protein, e.g., derived from a pathogen, a tumor associated antigen, a cytokine, or combination thereof.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified 10 recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The modified recombinant virus includes, within a non-essential region of the virus genome, a heterologous DNA 15 sequence which encodes an antigenic protein (e.g., derived from a pathogen, a tumor associated antigen, a cytokine, or combination thereof) wherein the composition, when administered to a host, is capable of inducing an immunological response specific to the 20 protein encoded by the pathogen.

In a further aspect, the present invention relates to a method for expressing a gene product in a cell cultured in vitro by introducing into the cell a modified recombinant virus having attenuated virulence and 25 enhanced safety. The modified recombinant virus can include, within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein, e.g., derived from a pathogen, a tumor associated antigen, a cytokine, or combination thereof.

30 In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further contains 35 DNA from a heterologous source in a nonessential region of the virus genome. The DNA can code for a tumor associated antigen, a cytokine, or a combination thereof.

In particular, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor or by utilizing naturally host restricted viruses. The virus used according to the present invention is 5 advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus. Advantageously, the open reading frame is selected from the group consisting of J2R, B13R + B14R, A26L, A56R, C7L - K1L, and I4L (by the terminology 10 reported in Goebel et al., 1990a,b); and, the combination thereof. In this respect, the open reading frame comprises a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region or a large subunit, ribonucleotide 15 reductase; or, the combination thereof. The modified Copenhagen strain of vaccinia virus is identified as NYVAC (Tartaglia et al., 1992).

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description, given by way of 20 example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings, in which:

FIG. 1 schematically shows a method for the 25 construction of plasmid pSD460 for deletion of thymidine kinase gene and generation of recombinant vaccinia virus vP410;

FIG. 2 schematically shows a method for the 30 construction of plasmid pSD486 for deletion of hemorrhagic region and generation of recombinant vaccinia virus vP553;

FIG. 3 schematically shows a method for the 35 construction of plasmid pMP494Δ for deletion of ATI region and generation of recombinant vaccinia virus vP618;

FIG. 4 schematically shows a method for the construction of plasmid pSD467 for deletion of

hemagglutinin gene and generation of recombinant vaccinia virus vP723;

5 FIG. 5 schematically shows a method for the construction of plasmid pMPCK1Δ for deletion of gene cluster [C7L - K1L] and generation of recombinant vaccinia virus vP804;

10 FIG. 6 schematically shows a method for the construction of plasmid pSD548 for deletion of large subunit, ribonucleotide reductase and generation of recombinant vaccinia virus vP866 (NYVAC);

15 FIG. 7 schematically shows a method for the construction of plasmid pRW842 for insertion of rabies glycoprotein G gene into the TK deletion locus and generation of recombinant vaccinia virus vP879;

FIG. 8 shows the DNA sequence (SEQ ID NO:68) of a canarypox PvuII fragment containing the C5 ORF.

FIGS. 9A and 9B schematically show a method for the construction of recombinant canarypox virus vCP65 (ALVAC-RG);

20 FIG. 10 shows schematically the ORFs deleted to generate NYVAC;

FIG. 11 shows the nucleotide sequence (SEQ ID NO:77) of a fragment of TROVAC DNA containing an F8 ORF;

25 FIG. 12 shows the DNA sequence (SEQ ID NO:78) of a 2356 base pair fragment of TROVAC DNA containing the F7 ORF;

30 FIGS. 13A to 13D show graphs of rabies neutralizing antibody titers (RFFIT, IU/ml), booster effect of HDC and vCP65 ($10^{5.5}$ TCID50) in volunteers previously immunized with either the same or the alternate vaccine (vaccines given at days 0, 28 and 180, antibody titers measured at days 0, 7, 28, 35, 56, 173, 187 and 208);

35 FIG. 14A to 14C show the nucleotide sequence of a 7351 bp fragment containing the ALVAC C3 insertion site (SEQ ID NO:127);

FIG. 15 shows the nucleotide sequences of H6/TNF- α expression cassette and flanking regions from vCP245 (SEQ

ID NO:79);

FIG. 16 shows the nucleotide sequence of the H6/TNF- α expression cassette and flanking regions from vP1200 (SEQ ID NO:89);

5 FIG. 17 shows the nucleotide sequence of the H6/p53 (wildtype) expression cassette and flanking regions from vP1101 (SEQ ID NO:99);

10 FIG. 18 shows the nucleotide sequence of the H6/p53 (wildtype) expression cassette and flanking regions from vCP207 (SEQ ID NO:99);

FIG. 19 shows the nucleotide sequence of the H6/MAGE-1 expression cassette and flanking region from vCP235 (SEQ ID NO:109);

15 FIG. 20 shows the nucleotide sequence of the H6/MAGE-1 expression cassette and flanking regions from pMAW037 (SEQ ID NO:110);

FIG. 21A and B show the nucleotide sequence of the p126.15 SERA cDNA insert along with the predicted amino acid sequence (SEQ ID NOS:119; 120);

20 FIG. 22 shows the nucleotide sequence of the H6/CEA expression cassette and flanking regions from pH6.CEA.C3.2 (SEQ ID NO:144);

FIG. 23 shows the nucleotide sequence of the H6/CEA expression cassette and flanking regions from pH6.CEA.HA (SEQ ID NO:145);

25 FIG. 24 shows the nucleotide sequence of murine IL-2 from the translation initiation codon through the stop codon (SEQ ID NO:150);

FIG. 25 shows the corrected nucleotide sequence of 30 human IL-2 from the translation initiation codon through the stop codon (SEQ ID NO:159);

FIG. 26 shows the nucleotide sequence of the I3L/murine IFN γ expression cassette (SEQ ID NO:163);

35 FIG. 27 shows the nucleotide sequence of the I3L/human IFN γ expression cassette (SEQ ID NO:168);

FIG. 28 shows the nucleotide sequence of the canarypox insert in pC6HIII3kb (SEQ ID NO:169);

FIG. 29 shows the nucleotide sequence pC6L (SEQ ID NO:174);

FIG. 30 shows the nucleotide sequence of the E3L/murine IL-4 expression cassette (SEQ ID NO:178);

5 FIG. 31 shows the nucleotide sequence of the expression cassette comprising the E3L promoted human IL-4 gene (SEQ ID NO:186);

FIG. 32 shows the nucleotide sequence of the vaccinia E3L/hGMCSF expression cassette (SEQ ID NO:191);

10 FIG. 33 shows the sequence of the EPV 42kDa/human IL-12 P40 expression cassette (SEQ ID NO:194);

FIG. 34 shows the nucleotide sequence of the vaccinia E3L/human IL-12 P35 expression cassette (SEQ ID NO:199);

15 FIG. 35 shows the nucleotide sequence of the murine B7 gene (SEQ ID NO:202);

FIG. 36 shows flow cytometric analysis of murine B7 expression in NYVAC and ALVAC infected murine tumor cell lines;

20 FIG. 37 shows the nucleotide sequence for the human B7 gene (SEQ ID NO:207);

FIG. 38 shows the murine p53 gene (SEQ ID NO:214); and

25 FIG. 39 shows the coding sequence for the human p53 gene (SEQ ID NO:215).

DETAILED DESCRIPTION OF THE INVENTION

To develop a new vaccinia vaccine strain, NYVAC (vP866), the Copenhagen vaccine strain of vaccinia virus was modified by the deletion of six nonessential regions 30 of the genome encoding known or potential virulence factors. The sequential deletions are detailed below. All designations of vaccinia restriction fragments, open reading frames and nucleotide positions are based on the terminology reported in Goebel et al., 1990a,b.

35 The deletion loci were also engineered as recipient loci for the insertion of foreign genes.

The regions deleted in NYVAC are listed below. Also

listed are the abbreviations and open reading frame designations for the deleted regions (Goebel et al., 1990a,b) and the designation of the vaccinia recombinant (vP) containing all deletions through the deletion 5 specified:

- (1) thymidine kinase gene (TK; J2R) vP410;
- (2) hemorrhagic region (u; B13R + B14R) vP553;
- (3) A type inclusion body region (ATI; A26L) vP618;
- (4) hemagglutinin gene (HA; A56R) vP723;
- 10 (5) host range gene region (C7L - K1L) vP804; and
- (6) large subunit, ribonucleotide reductase (I4L) vP866 (NYVAC).

NYVAC is a genetically engineered vaccinia virus strain that was generated by the specific deletion of 15 eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC is highly attenuated by a number of criteria including i) decreased virulence after intracerebral inoculation in newborn mice, ii) inocuity in genetically (nu⁺/nu⁺) or 20 chemically (cyclophosphamide) immunocompromised mice, iii) failure to cause disseminated infection in immunocompromised mice, iv) lack of significant induration and ulceration on rabbit skin, v) rapid 25 clearance from the site of inoculation, and vi) greatly reduced replication competency on a number of tissue culture cell lines including those of human origin. Nevertheless, NYVAC based vectors induce excellent 30 responses to extrinsic immunogens and provided protective immunity.

30 TROVAC refers to an attenuated fowlpox that was a plaque-cloned isolate derived from the FP-1 vaccine strain of fowlpoxvirus which is licensed for vaccination of 1 day old chicks. ALVAC is an attenuated canarypox 35 virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al., 1992). ALVAC has some general properties which are the same as some general properties of Kanapox. ALVAC-

based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors (Tartaglia et al., 1993 a,b). This avipox vector is restricted to avian species for productive 5 replication. On human cell cultures, canarypox virus replication is aborted early in the viral replication cycle prior to viral DNA synthesis. Nevertheless, when engineered to express extrinsic immunogens, authentic expression and processing is observed *in vitro* in 10 mammalian cells and inoculation into numerous mammalian species induces antibody and cellular immune responses to the extrinsic immunogen and provides protection against challenge with the cognate pathogen (Taylor et al., 1992; Taylor et al., 1991). Recent Phase I clinical trials in 15 both Europe and the United States of a canarypox/rabies glycoprotein recombinant (ALVAC-RG) demonstrated that the experimental vaccine was well tolerated and induced protective levels of rabiesvirus neutralizing antibody titers (Cadoz et al., 1992; Fries et al., 1992). 20 Additionally, peripheral blood mononuclear cells (PBMCs) derived from the ALVAC-RG vaccine demonstrated significant levels of lymphocyte proliferation when stimulated with purified rabies virus (Fries et al., 1992). 25 NYVAC, ALVAC and TROVAC have also been recognized as unique among all poxviruses in that the National Institutes of Health ("NIH") (U.S. Public Health Service), Recombinant DNA Advisory Committee, which issues guidelines for the physical containment of genetic 30 material such as viruses and vectors, i.e., guidelines for safety procedures for the use of such viruses and vectors which are based upon the pathogenicity of the particular virus or vector, granted a reduction in physical containment level: from BL2 to BL1. No other 35 poxvirus has a BL1 physical containment level. Even the Copenhagen strain of vaccinia virus - the common smallpox vaccine - has a higher physical containment level;

namely, BL2. Accordingly, the art has recognized that NYVAC, ALVAC and TROVAC have a lower pathogenicity than any other poxvirus.

Both NYVAC- and ALVAC-based recombinant viruses have been shown to stimulate *in vitro* specific CD8⁺ CTLs from human PBMCs (Tartaglia et al., 1993a). Mice immunized with NYVAC or ALVAC recombinants expressing various forms of the HIV-1 envelope glycoprotein generated both primary and memory HIV specific CTL responses which could be recalled by a second inoculation (Tartaglia et al., 1993a). ALVAC-env and NYVAC-env recombinants (expressing the HIV-1 envelope glycoprotein) stimulated strong HIV-specific CTL responses from peripheral blood mononuclear cells (PBMC) of HIV-1 infected individuals (Tartaglia et al., 1993a). Acutely infected autologous PBMC were used as stimulator cells for the remaining PBMC. After 10 days incubation in the absence of exogenous IL-2, the cells were evaluated for CTL activities. NYVAC-env and ALVAC-env stimulated high levels of anti-HIV activities. Thus, these vectors lend themselves well to *ex vivo* stimulation of antigen reactive lymphocytes; for example, adoptive immunotherapy such as the *ex vivo* expression of tumor reactive lymphocytes and reinfusion into the host (patient).

Immunization of the patient with NYVAC-, ALVAC-, or TROVAC-based recombinant viruses expressing TAAs produced by the patient's tumor cells can elicit anti-tumor immune responses more rapidly and to sufficient levels to impede or halt tumor spread and potentially eliminate the tumor burden.

Clearly based on the attenuation profiles of the NYVAC, ALVAC, and TROVAC vectors and their demonstrated ability to elicit both humoral and cellular immunological responses to extrinsic immunogens (Tartaglia et al., 1993a,b; Taylor et al., 1992; Konishi et al., 1992) such recombinant viruses offer a distinct advantage over previously described vaccinia-based recombinant viruses.

The immunization procedure for such recombinant viruses as immunotherapeutic vaccines or compositions may be via a parenteral route (intradermal, intramuscular or subcutaneous). Such an administration enables a systemic 5 immune response against the specific TAA(s). Alternatively, the vaccine or composition may be administered directly into the tumor mass (intratumor). Such a route of administration can enhance the anti-tumor activities of lymphocytes specifically associated with 10 tumors (Rosenberg, 1992). Immunization of the patient with NYVAC-, ALVAC- or TROVAC-based recombinant viruses expressing TAAs produced by the patient's tumor cells can elicit anti-tumor immune responses more rapidly and to sufficient levels to impede or halt tumor spread and 15 potentially eliminate the tumor burden. The heightened tumor-specific immune response resulting from vaccinations with these poxvirus-based recombinant vaccines can result in remission of the tumor, including permanent remission of the tumor. Examples of known TAAs 20 for which recombinant poxviruses can be generated and employed with immunotherapeutic value in accordance with this invention include, but are not limited to p53 (Hollstein et al., 1991), p21-ras (Almoguera et al., 1988), HER-2 (Fendly et al., 1990), and the melanoma- 25 associated antigens (MAGE-1; MZE-2) (van der Bruggen et al., 1991), and p97 (Hu et al., 1988) and the carcinoembryonic antigen (CEA) associated with colorectal cancer (Kantor et al., 1993; Fishbein et al., 1992; Kaufman et al., 1991).

30 More generally, the inventive vaccines or compositions (vaccines or compositions containing the poxvirus recombinants of the invention) can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such vaccines 35 or compositions can be administered to a patient in need of such administration in dosages and by techniques well known to those skilled in the medical arts taking into

consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration. The vaccines or compositions can be co-administered or sequentially administered with other 5 antineoplastic, anti-tumor or anti-cancer agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor or anti-cancer agents; again taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and, the 10 route of administration.

Examples of vaccines or compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, etc., administration such as suspensions, syrups or elixirs; and, preparations for 15 parental, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the recombinant poxvirus may be in admixture with a suitable carrier, diluent, or excipient 20 such as sterile water, physiological saline, glucose or the like. The recombinant poxvirus of the invention can be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline buffer. Further, the invention also comprehends a kit wherein the 25 recombinant poxvirus is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can also include an additional anti-cancer, anti-tumor or antineoplastic agent and/or an agent which reduces or alleviates ill effects of 30 antineoplastic, anti-tumor or anti-cancer agents for co- or sequential-administration. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

The poxvirus vector technology provides an appealing 35 approach towards manipulating lymphocytes and tumor cells for use in cell-based immunotherapeutic modalities for cancer. Characteristics of the NYVAC, ALVAC and TROVAC

vectors providing the impetus for such applications include 1) their apparent independence for specific receptors for entry into cells, 2) their ability to express foreign genes in cell substrates despite their 5 species- or tissue-specific origin, 3) their ability to express foreign genes independent of host cell regulation, 4) the demonstrated ability of using poxvirus recombinant viruses to amplify specific CTL reactivities from peripheral blood mononuclear cells (PBMCS), and 5) 10 their highly attenuated properties compared to existing vaccinia virus vaccine strains (Reviewed by Tartaglia et al., 1993a; Tartaglia et al., 1990).

The expression of specific cytokines or the co-expression of specific cytokines with TAAs by NYVAC-, 15 ALVAC-, and TROVAC-based recombinant viruses can enhance the numbers and anti-tumor activities of CTLs associated with tumor cell depletion or elimination. Examples of cytokines which have a beneficial effect in this regard include tumor necrosis factor- α (TNF- α), interferon-gamma 20 (INF-gamma), interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-7 (IL-7) (reviewed by Pardoll, 1992). Cytokine interleukin 2 (IL-2) plays a major role in 25 promoting cell mediated immunity. Secreted by the T_H1 subset of lymphocytes, IL-2 is a T cell growth factor which stimulates division of both CD4 $^{+}$ and CD8 $^{+}$ T cells. In addition, IL-2 also has been shown to activate B cells, monocytes and natural killer cells. To a large degree the biological effects of IL-2 are due to its role 30 in inducing production of IFN γ . Recombinant vaccinia virus expressing IL-2 is attenuated in mice compared to wild-type vaccinia virus. This is due to the ability of the vaccinia-expressed IL-2 to stimulate mouse NK cells 35 to produce IFN γ , which limits the growth of the recombinant vaccinia virus (Karupiah et al., 1990). Similarly, it has been shown that inoculation of immunodeficient athymic nude mice with recombinant vaccinia virus expressing both IL-2 and the HA gene of

influenza can protect these mice from subsequent challenge with influenza virus (Karupiah et al., 1992).

Cytokine interferon γ (IFN γ) is secreted by the T_H1 subset of lymphocytes. IFN γ promotes the T_H1 cell mediated immune response, while inhibiting the T_H2 (antibody) response. IFN γ induces the expression of major histocompatibility complex (MHC) molecules on antigen presenting cells, and induces the expression of the B7 costimulatory molecule on macrophages. In addition to enhancing the phagocytic activity of macrophages, IFN γ enhances the cytotoxic activity of NK cells. When expressed in replicating recombinant vaccinia virus, IFN γ limits the growth of the recombinant virus. This allows T cell immunodeficient mice to resolve the infection (Kohonen-Corish et al., 1990).

Cytokine interleukin 4 (IL-4) is secreted by the T_H2 subset of lymphocytes. IL-4 promotes the T_H2 (antibody) response, while inhibiting the T_H1 cell mediated immune response. Recombinant vaccinia virus expressing IL-4 shows increased pathogenicity in mice compared to wild-type vaccinia virus (Ramshaw et al., 1992).

Cytokine granulocyte macrophage colony stimulating factor (GMCSF) is pleiotropic. In addition to stimulating the proliferation of cells of both the granulocyte and macrophage cell lineages, GMCSF, in cross-competition with interleukins 3 and 5 (IL-3 and IL-5), influences many other aspects of hematopoiesis and may play a role in facilitation of tumor cell growth (Lopez et al., 1992). GMCSF is used clinically for hematopoietic reconstitution following bone marrow transplantation.

Cytokine interleukin 12 (IL-12), formerly known as natural killer (NK) cell stimulatory factor, is a heterodimer composed of 35kDa and 40kDa subunits. IL-12 is produced by monocytes, macrophages, B cells and other accessory cells. IL-12 has pleiotropic effects on both NK cells and T cells. Partly through its role in

inducing IFN γ production, IL-12 plays a major role in promoting the T_H1 cell mediated immune response, while inhibiting the T_H2 response (reviewed in Trinchieri, 1993). Recently, recombinant murine IL-12 has been 5 demonstrated to have potent antitumor and antimetastatic effects in mice (Brunda et al., 1993).

B7(BB-1), a member of the immunoglobulin superfamily, is present on the surface of antigen presenting cells. Interaction of the B7 molecule on antigen presenting 10 cells with its receptors on T cells provides costimulatory signals, including IL-2, which are necessary for T cell activation (Schwartz, 1992). Recently it was shown that experimental co-expression of B7 along with a tumor antigen on murine melanoma cells 15 can lead to regression of tumors in mice. This was accomplished by the B7-assisted activation of tumor-specific cytotoxic T cells (Chen et al., 1992).

The c-erb-B-2 gene, which is conserved among vertebrates, encodes a possible receptor protein. The 20 185 kDa translation product contains a kinase domain which is highly homologous to the kinase domain of the epidermal growth factor (EGF) receptor. The c-erb-B-2 gene is conserved among vertebrates, and is the same as the rat neu gene, which has been detected in a number of 25 rat neuro/glioblastomas. The human c-erb-B-2 gene, also known as HER2, is amplified in certain neoplasias, most notably breast cancer. In the gastric cancer cell line, MKN-7, both the normal 4.6 kb transcript encoding c-erb-B-2 and a 2.3 kb transcript which specifies only the 30 extracellular domain of the putative receptor are synthesized at elevated levels (Yamamoto et al., 1986). The extracellular domain has been suggested as a potential immunogen for active specific immunotherapy of breast cancer (Fendly et al., 1990).

35 Utility of NYVAC-, ALVAC-, and TROVAC-based recombinant viruses expressing TAAs plus or minus specific cytokines for adoptive immunotherapy can take

several forms. For one, genetic modification of PBMCS can be accomplished by vector-mediated introduction of TAAs, cytokine genes, or other genes and then directly reintroduced into the patient. Such administration 5 relies on the drainage or movement of modified PBMCS to lymphoid tissue (i.e. spleen; lymph nodes) via the reticuloendothelial system (RES) for elicitation of the tumor-specific immune response. PBMCS modified by infection with the pertinent NYVAC-, ALVAC-, and TROVAC- 10 based recombinant can be employed, for instance, *in vitro*, to expand TAA-specific CTLs for reinfusion into the patient. Tumor-infiltrating lymphocytes (TILs) derived from the tumor mass can be isolated, expanded, and modified to express pertinent genes using NYVAC-, 15 ALVAC-, or TROVAC-based recombinants viruses prior to reinfusion into the patient. TILs retain the capability of returning to tumors (homing) when re-introduced into the subject (Rosenberg, 1992). Thus, they provide a 20 convenient vehicle for delivery of cytotoxic or cytostatic cytokines to tumor masses.

Cell-based active immunotherapy can also take on several potential modalities using the NYVAC-, ALVAC-, and TROVAC vectors. Tumor cells can be modified to express TAAs, cytokines, or other novel antigens (i.e. 25 class I or class II major histocompatibility genes). Such modified tumor cells can subsequently be utilized for active immunization. The therapeutic potential for such an administration is based on the ability of these modified tumor cells to secrete cytokines and to alter 30 the presentation of TAAs to achieve systemic anti-tumor activity. The modified tumor cells can also be utilized to expand tumor-specific CTLs *in vitro* for reinfusion into the patient.

A better understanding of the present invention and 35 of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

DNA Cloning and Synthesis. Plasmids were constructed, screened and grown by standard procedures (Maniatis et al., 1982; Perkus et al., 1985; Piccini et al., 1987). Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, MD, New England Biolabs, Beverly, MA; and Boehringer Mannheim Biochemicals, Indianapolis, IN. Klenow fragment of *E. coli* polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England Biolabs. The reagents were used as specified by the various suppliers.

Synthetic oligodeoxyribonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as previously described (Perkus et al., 1989). DNA sequencing was performed by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase (Tabor et al., 1987) as previously described (Guo et al., 1989). DNA amplification by polymerase chain reaction (PCR) for sequence verification (Engelke et al., 1988) was performed using custom synthesized oligonucleotide primers and GeneAmp DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) in an automated Perkin Elmer Cetus DNA Thermal Cycler. Excess DNA sequences were deleted from plasmids by restriction endonuclease digestion followed by limited digestion by BAL-31 exonuclease and mutagenesis (Mandecki, 1986) using synthetic oligonucleotides.

Cells, Virus, and Transfection. The origins and conditions of cultivation of the Copenhagen strain of vaccinia virus has been previously described (Guo et al., 1989). Generation of recombinant virus by recombination, *in situ* hybridization of nitrocellulose filters and screening for B-galactosidase activity are as previously described (Piccini et al., 1987).

The origins and conditions of cultivation of the Copenhagen strain of vaccinia virus and NYVAC has been

previously described (Guo et al., 1989; Tartaglia et al., 1992). Generation of recombinant virus by recombination, *in situ* hybridization of nitrocellulose filters and screening for B-galactosidase activity are as previously 5 described (Panicali et al., 1982; Perkus et al., 1989).

The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo 10 fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in *in vitro* recombination tests. The plaque 15 purified canarypox isolate is designated ALVAC.

The strain of fowlpox virus (FPV) designated FP-1 has been described previously (Taylor et al., 1988a). It is an attenuated vaccine strain useful in vaccination of day old chickens. The parental virus strain Duvette was 20 obtained in France as a fowlpox scale from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chicken embryo fibroblast cells. The virus was subjected to four successive plaque purifications. 25 One plaque isolate was further amplified in primary CEF cells and a stock virus, designated as TROVAC, established.

NYVAC, ALVAC and TROVAC viral vectors and their derivatives were propagated as described previously 30 (Piccini et al., 1987; Taylor et al., 1988a,b). Vero cells and chick embryo fibroblasts (CEF) were propagated as described previously (Taylor et al., 1988a,b).

Example 1 - CONSTRUCTION OF PLASMID PSD460 FOR
DELETION OF THYMIDINE KINASE GENE (J2R)

35 Referring now to FIG. 1, plasmid PSD406 contains vaccinia HindIII J (pos. 83359 - 88377) cloned into pUC8. PSD406 was cut with HindIII and PvuII, and the 1.7 kb

fragment from the left side of HindIII J cloned into pUC8 cut with HindIII/SmaI, forming pSD447. pSD447 contains the entire gene for J2R (pos. 83855 - 84385). The initiation codon is contained within an NlaIII site and the termination codon is contained within an SspI site. Direction of transcription is indicated by an arrow in FIG. 1.

To obtain a left flanking arm, a 0.8 kb HindIII/EcoRI fragment was isolated from pSD447, then digested with NlaIII and a 0.5 kb HindIII/NlaIII fragment isolated. Annealed synthetic oligonucleotides MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ ID NO:2)

		<u>Sma</u> I	
MPSYN43	5'	TAATTAACCTAGCTACCCGGG	3'
15	MPSYN44	3'	GTACATTAATTGATCGATGGGCCCTTAA
		<u>Nla</u> III	<u>Eco</u> RI

were ligated with the 0.5 kb HindIII/NlaIII fragment into pUC18 vector plasmid cut with HindIII/EcoRI, generating plasmid pSD449.

To obtain a restriction fragment containing a vaccinia right flanking arm and pUC vector sequences, pSD447 was cut with SspI (partial) within vaccinia sequences and HindIII at the pUC/vaccinia junction, and a 2.9 kb vector fragment isolated. This vector fragment was ligated with annealed synthetic oligonucleotides MPSYN45/MPSYN46 (SEQ ID NO:3/SEQ ID NO:4)

		<u>Hind</u> III <u>Sma</u> I	
MPSYN45	5'	AGCTTCCCGGGTAAGTAATACGTCAAGGAGAAAACGAA	3'
30	MPSYN46	3'	AGGGCCCATTATGCAGTCCTCTTTGCTT

		<u>Not</u> I		<u>Ssp</u> I	
		ACGATCTGTAGTTAGCGGGCGCTAATTAACATAAT	3'	MPSYN45	
		TGCTAGACATCAATGCCGGCGGATTAATTGATTA	5'	MPSYN46	
35					

generating pSD459.

To combine the left and right flanking arms into one plasmid, a 0.5 kb HindIII/SmaI fragment was isolated from pSD449 and ligated with pSD459 vector plasmid cut with HindIII/SmaI, generating plasmid pSD460. pSD460 was used as donor plasmid for recombination with wild type parental vaccinia virus Copenhagen strain VC-2. ³²P

labelled probe was synthesized by primer extension using MPSYN45 (SEQ ID NO:3) as template and the complementary 20mer oligonucleotide MPSYN47 (SEQ ID NO:5) (5' TTAGTTAATTAGGCGGCCGC 3') as primer. Recombinant 5 virus vP410 was identified by plaque hybridization.

Example 2 - CONSTRUCTION OF PLASMID psd486 FOR
DELETION OF HEMORRHAGIC REGION
(B13R + B14R)

Referring now to FIG. 2, plasmid pSD419 contains
10 vaccinia SalI G (pos. 160,744-173,351) cloned into pUC8.
pSD422 contains the contiguous vaccinia SalI fragment to
the right, SalI J (pos. 173,351-182,746) cloned into
pUC8. To construct a plasmid deleted for the hemorrhagic
region, u, B13R - B14R (pos. 172,549 - 173,552), pSD419
15 was used as the source for the left flanking arm and
pSD422 was used as the source of the right flanking arm.
The direction of transcription for the u region is
indicated by an arrow in FIG. 2.

To remove unwanted sequences from pSD419, sequences
20 to the left of the NcoI site (pos. 172,253) were removed
by digestion of pSD419 with NcoI/SmaI followed by blunt
ending with Klenow fragment of *E. coli* polymerase and
ligation generating plasmid pSD476. A vaccinia right
flanking arm was obtained by digestion of pSD422 with
25 HpaI at the termination codon of B14R and by digestion
with NruI 0.3 kb to the right. This 0.3 kb fragment was
isolated and ligated with a 3.4 kb HincII vector fragment
isolated from pSD476, generating plasmid pSD477. The
location of the partial deletion of the vaccinia u region
30 in pSD477 is indicated by a triangle. The remaining B13R
coding sequences in pSD477 were removed by digestion with
ClaI/HpaI, and the resulting vector fragment was ligated
with annealed synthetic oligonucleotides SD22mer/SD20mer
(SEQ ID NO:6/SEQ ID NO:7)

35	SD22mer	5'	<u>Cla</u> I	BamHI	<u>Hpa</u> I	3'
			CGATTACTAT <u>G</u> AAGGATCCGTT			
			SD20mer	3'	TAATGATACTTCCTAGGCAA	5'

generating pSD479. pSD479 contains an initiation codon

(underlined) followed by a BamHI site. To place *E. coli* Beta-galactosidase in the B13-B14 (u) deletion locus under the control of the u promoter, a 3.2 kb BamHI fragment containing the Beta-galactosidase gene (Shapira et al., 1983) was inserted into the BamHI site of pSD479, generating pSD479BG. pSD479BG was used as donor plasmid for recombination with vaccinia virus vP410. Recombinant vaccinia virus vP533 was isolated as a blue plaque in the presence of chromogenic substrate X-gal. In vP533 the B13R-B14R region is deleted and is replaced by Beta-galactosidase.

To remove Beta-galactosidase sequences from vP533, plasmid pSD486, a derivative of pSD477 containing a polylinker region but no initiation codon at the u deletion junction, was utilized. First the ClaI/HpaI vector fragment from pSD477 referred to above was ligated with annealed synthetic oligonucleotides SD42mer/SD40mer (SEQ ID NO:8/SEQ ID NO:9)

ClaI SacI XbaI HpaI
20 SD42mer 5' CGATTACTAGATCTGAGCTCCCCGGGCTCGAGGGATCCGTT 3'
SD40mer 3' TAATGATCTAGACTCGAGGGGCCGAGCTCCCTAGGCAA 5'
 BglII SmaI BamHI

generating plasmid pSD478. Next the EcoRI site at the pUC/vaccinia junction was destroyed by digestion of pSD478 with EcoRI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation, generating plasmid pSD478E⁻. pSD478E⁻ was digested with BamHI and HpaI and ligated with annealed synthetic oligonucleotides HEM5/HEM6 (SEQ ID NO:10/SEQ ID NO:11)

BamHI EcoRI HpaI
HEM5 5' GATCCGAATTCTAGCT 3'
HEM6 3' GCTTAAGATCGA 5'

35 generating plasmid pSD486. pSD486 was used as donor plasmid for recombination with recombinant vaccinia virus vP533, generating vP553, which was isolated as a clear plaque in the presence of X-gal.

Example 3 - CONSTRUCTION OF PLASMID pMP494Δ FOR DELETION OF ATI REGION (A26L)

Referring now to FIG. 3, pSD414 contains SalI B cloned into pUC8. To remove unwanted DNA sequences to the left of the A26L region, pSD414 was cut with XbaI within vaccinia sequences (pos. 137,079) and with HindIII at the pUC/vaccinia junction, then blunt ended with Klenow fragment of *E. coli* polymerase and ligated, resulting in plasmid pSD483. To remove unwanted vaccinia DNA sequences to the right of the A26L region, pSD483 was cut with EcoRI (pos. 140,665 and at the pUC/vaccinia junction) and ligated, forming plasmid pSD484. To remove the A26L coding region, pSD484 was cut with NdeI (partial) slightly upstream from the A26L ORF (pos. 139,004) and with HpaI (pos. 137,889) slightly downstream from the A26L ORF. The 5.2 kb vector fragment was isolated and ligated with annealed synthetic oligonucleotides ATI3/ATI4 (SEQ ID NO:12/SEQ ID NO:13)

20 NdeI
ATI3 5' TATGAGTAACTTAACTCTTTGTTAATTAAAAGTATATTCAAAAAATAAGT
ATI4 3' ACTCATTGAATTGAGAAAACAATTAAATTTCATATAAGTTTTATTCA

25 BglII EcoRI HpaI
TATATAAATAGATCTGAATTCGTT 3' ATI3
ATATATTATCTAGACTTAAGCAA 5' ATI4

reconstructing the region upstream from A26L and replacing the A26L ORF with a short polylinker region containing the restriction sites BglII, EcoRI and HpaI, as indicated above. The resulting plasmid was designated pSD485. Since the BglII and EcoRI sites in the polylinker region of pSD485 are not unique, unwanted BglII and EcoRI sites were removed from plasmid pSD483 (described above) by digestion with BglII (pos. 140,136) and with EcoRI at the pUC/vaccinia junction, followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation. The resulting plasmid was designated pSD489. The 1.8 kb ClaI (pos. 137,198)/EcoRV (pos. 139,048) fragment from pSD489 containing the A26L ORF was replaced with the corresponding 0.7 kb polylinker-

containing ClaI/EcoRV fragment from pSD485, generating pSD492. The BglII and EcoRI sites in the polylinker region of pSD492 are unique.

A 3.3 kb BglII cassette containing the *E. coli* Beta-5 galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Perkus et al., 1990) was inserted into the BglII site of pSD492, forming pSD493KBG. Plasmid pSD493KBG was used in recombination with rescuing virus 10 vP553. Recombinant vaccinia virus, vP581, containing Beta-galactosidase in the A26L deletion region, was isolated as a blue plaque in the presence of X-gal.

To generate a plasmid for the removal of Beta-galactosidase sequences from vaccinia recombinant virus 15 vP581, the polylinker region of plasmid pSD492 was deleted by mutagenesis (Mandecki, 1986) using synthetic oligonucleotide MPSYN177 (SEQ ID NO:14) (5' AAAATGGCGTGGATTGTTAACTTATATAACTTATTTTTGAATATAC 3'). In the resulting plasmid, pMP494Δ, vaccinia DNA encompassing 20 positions [137,889 - 138,937], including the entire A26L ORF is deleted. Recombination between the pMP494Δ and the Beta-galactosidase containing vaccinia recombinant, vP581, resulted in vaccinia deletion mutant vP618, which was isolated as a clear plaque in the presence of X-gal.

25 **Example 4 - CONSTRUCTION OF PLASMID pSD467 FOR DELETION OF HEMAGGLUTININ GENE (A56R)**

Referring now to FIG. 4, vaccinia SalI G restriction fragment (pos. 160,744-173,351) crosses the HindIII A/B 30 junction (pos. 162,539). pSD419 contains vaccinia SalI G cloned into pUC8. The direction of transcription for the hemagglutinin (HA) gene is indicated by an arrow in FIG. 4. Vaccinia sequences derived from HindIII B were removed by digestion of pSD419 with HindIII within 35 vaccinia sequences and at the pUC/vaccinia junction followed by ligation. The resulting plasmid, pSD456, contains the HA gene, A56R, flanked by 0.4 kb of vaccinia sequences to the left and 0.4 kb of vaccinia sequences to

the right. A56R coding sequences were removed by cutting pSD456 with RsaI (partial; pos. 161,090) upstream from A56R coding sequences, and with EagI (pos. 162,054) near the end of the gene. The 3.6 kb RsaI/EagI vector 5 fragment from pSD456 was isolated and ligated with annealed synthetic oligonucleotides MPSYN59 (SEQ ID NO:15), MPSYN62 (SEQ ID NO:16), MPSYN60 (SEQ ID NO:17), and MPSYN61 (SEQ ID NO:18)

RsaI
10 MPSYN59 5' ACACGAATGATTTCTAAAGTATTGGAAAGTTTATAGGT-
MPSYN62 3' TGTGCTTACTAAAAGATTCATAAACCTTCAAAATATCCA-
MPSYN59 AGTTGATAGAACAAAATACATAATTT 3'
MPSYN62 TCAACTATCT 5'
15 MPSYN60 5' TGTAAAAATAATCACTTTTATA-
MPSYN61 3' TGTTTTATGTATTAAAACATTTTATTAGTGAAAAATAT-
BglII SmaI PstI EagI
20 MPSYN60 CTAAGATCTCCCGGGCTGCAGC 3'
MPSYN61 GATTCTAGAGGGCCCGACGTCGCCGG 5'

reconstructing the DNA sequences upstream from the A56R ORF and replacing the A56R ORF with a polylinker region 25 as indicated above. The resulting plasmid is pSD466. The vaccinia deletion in pSD466 encompasses positions [161,185-162,053]. The site of the deletion in pSD466 is indicated by a triangle in FIG. 4.

A 3.2 kb BglIII/BamHI (partial) cassette containing 30 the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Guo et al., 1989) was inserted into the BglIII site of pSD466, forming pSD466KBG. Plasmid pSD466KBG was used in recombination with rescuing 35 virus vP618. Recombinant vaccinia virus, vP708, containing Beta-galactosidase in the A56R deletion, was isolated as a blue plaque in the presence of X-gal.

Beta-galactosidase sequences were deleted from vP708 using donor plasmid pSD467. pSD467 is identical to 40 pSD466, except that EcoRI, SmaI and BamHI sites were removed from the pUC/vaccinia junction by digestion of pSD466 with EcoRI/BamHI followed by blunt ending with

Klenow fragment of *E. coli* polymerase and ligation. Recombination between vP708 and pSD467 resulted in recombinant vaccinia deletion mutant, vP723, which was isolated as a clear plaque in the presence of X-gal.

5 **Example 5 - CONSTRUCTION OF PLASMID pMPCSK1Δ FOR DELETION OF OPEN READING FRAMES [C7L-K1L]**

Referring now to FIG. 5, the following vaccinia 10 clones were utilized in the construction of pMPCSK1Δ. pSD420 is SalI H cloned into pUC8. pSD435 is KpnI F cloned into pUC18. pSD435 was cut with SphI and religated, forming pSD451. In pSD451, DNA sequences to the left of the SphI site (pos. 27,416) in HindIII M are 15 removed (Perkus et al., 1990). pSD409 is HindIII M cloned into pUC8.

To provide a substrate for the deletion of the [C7L-K1L] gene cluster from vaccinia, *E. coli* Beta-galactosidase was first inserted into the vaccinia M2L 20 deletion locus (Guo et al., 1990) as follows. To eliminate the BglII site in pSD409, the plasmid was cut with BglII in vaccinia sequences (pos. 28,212) and with BamHI at the pUC/vaccinia junction, then ligated to form 25 plasmid pMP409B. pMP409B was cut at the unique SphI site (pos. 27,416). M2L coding sequences were removed by mutagenesis (Guo et al., 1990; Mandecki, 1986) using synthetic oligonucleotide

30 BglII
MPSYN82 (SEQ ID NO:19) 5' TTTCTGTATTTGCACCAATTAGATCTT-
ACTCAAAATATGTAACAAATA 3'

The resulting plasmid, pMP409D, contains a unique BglII site inserted into the M2L deletion locus as indicated above. A 3.2 kb BamHI (partial)/BglII cassette 35 containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the 11 kDa promoter (Bertholet et al., 1985) was inserted into pMP409D cut with BglII. The resulting plasmid, pMP409DBG (Guo et al., 1990), was used as donor plasmid for recombination 40 with rescuing vaccinia virus vP723. Recombinant vaccinia

virus, vP784, containing Beta-galactosidase inserted into the M2L deletion locus, was isolated as a blue plaque in the presence of X-gal.

A plasmid deleted for vaccinia genes [C7L-K1L] was 5 assembled in pUC8 cut with SmaI, HindIII and blunt ended with Klenow fragment of *E. coli* polymerase. The left flanking arm consisting of vaccinia HindIII C sequences was obtained by digestion of pSD420 with XbaI (pos. 18,628) followed by blunt ending with Klenow fragment of 10 *E. coli* polymerase and digestion with BglII (pos. 19,706). The right flanking arm consisting of vaccinia HindIII K sequences was obtained by digestion of pSD451 with BglII (pos. 29,062) and EcoRV (pos. 29,778). The resulting plasmid, pMP581CK is deleted for vaccinia 15 sequences between the BglII site (pos. 19,706) in HindIII C and the BglII site (pos. 29,062) in HindIII K. The site of the deletion of vaccinia sequences in plasmid pMP581CK is indicated by a triangle in FIG. 5.

To remove excess DNA at the vaccinia deletion 20 junction, plasmid pMP581CK, was cut at the NcoI sites within vaccinia sequences (pos. 18,811; 19,655), treated with Bal-31 exonuclease and subjected to mutagenesis (Mandecki, 1986) using synthetic oligonucleotide MPSYN233 (SEQ ID NO:20) 25 5'-TGTCAATTAAACACTATACTCATATTAAATAAAATAATTTATT-3'. The resulting plasmid, pMPCSK1A, is deleted for vaccinia sequences positions 18,805-29,108, encompassing 12 vaccinia open reading frames [C7L - K1L]. Recombination between pMPCSK1A and the Beta-galactosidase containing 30 vaccinia recombinant, vP784, resulted in vaccinia deletion mutant, vP804, which was isolated as a clear plaque in the presence of X-gal.

Example 6 - CONSTRUCTION OF PLASMID pSD548 FOR DELETION OF LARGE SUBUNIT, RIBONUCLEOTIDE REDUCTASE (I4L)

Referring now to FIG. 6, plasmid pSD405 contains vaccinia HindIII I (pos. 63,875-70,367) cloned in pUC8. pSD405 was digested with EcoRV within vaccinia sequences

(pos. 67,933) and with SmaI at the pUC/vaccinia junction, and ligated, forming plasmid pSD518. pSD518 was used as the source of all the vaccinia restriction fragments used in the construction of pSD548.

5 The vaccinia I4L gene extends from position 67,371-65,059. Direction of transcription for I4L is indicated by an arrow in FIG. 6. To obtain a vector plasmid fragment deleted for a portion of the I4L coding sequences, pSD518 was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001) and blunt ended using Klenow 10 fragment of *E. coli* polymerase. This 4.8 kb vector fragment was ligated with a 3.2 kb SmaI cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa 15 promoter (Bertholet et al., 1985; Perkus et al., 1990), resulting in plasmid pSD524KBG. pSD524KBG was used as donor plasmid for recombination with vaccinia virus vP804. Recombinant vaccinia virus, vP855, containing Beta-galactosidase in a partial deletion of the I4L gene, 20 was isolated as a blue plaque in the presence of X-gal.

To delete Beta-galactosidase and the remainder of the I4L ORF from vP855, deletion plasmid pSD548 was constructed. The left and right vaccinia flanking arms were assembled separately in pUC8 as detailed below and 25 presented schematically in FIG. 6.

To construct a vector plasmid to accept the left vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides 518A1/518A2 (SEQ ID NO:21/SEQ ID NO:22)

30 BamHI RsaI
518A1 5' GATCCTGAGTACTTGTAAATATAATGATATATATTTCACTTATCTCAT
518A2 3' GACTCATGAAACATTATATTACTATATAAAAGTGAATAGAGTA

35 BglII EcoRI
TTGAGAATAAAAGATCTTAGG 3' 518A1
AACTCTTATTTCTAGAATCCTTAA 5' 518A2

forming plasmid pSD531. pSD531 was cut with RsaI (partial) and BamHI and a 2.7 kb vector fragment 40 isolated. pSD518 was cut with BglII (pos. 64,459)/ RsaI

(pos. 64,994) and a 0.5 kb fragment isolated. The two fragments were ligated together, forming pSD537, which contains the complete vaccinia flanking arm left of the I4L coding sequences.

5 To construct a vector plasmid to accept the right vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides
518B1/518B2 (SEQ ID NO:23/SEQ ID NO:24)

10 518B1 5' BamHI BglII Smal
518B2 3' GTCTAGAGGGCCCTTTTTAATAAATTGAAAAGTAATTATC-

15 RsaI EcoRI
GGATTTGACGTATGTAGCGTACTAGG 3' 518B1
CCTAAACTGCATACTACGCATGATCCTTAA 5' 518B2

forming plasmid pSD532. pSD532 was cut with RsaI (partial)/EcoRI and a 2.7 kb vector fragment isolated. pSD532 was cut with RsaI within vaccinia sequences (pos. 20 67,436) and EcoRI at the vaccinia/pUC junction, and a 0.6 kb fragment isolated. The two fragments were ligated together, forming pSD538, which contains the complete vaccinia flanking arm to the right of I4L coding sequences.

25 The right vaccinia flanking arm was isolated as a 0.6 kb EcoRI/BglII fragment from pSD538 and ligated into pSD537 vector plasmid cut with EcoRI/BglII. In the resulting plasmid, pSD539, the I4L ORF (pos. 65,047-67,386) is replaced by a polylinker region, which is 30 flanked by 0.6 kb vaccinia DNA to the left and 0.6 kb vaccinia DNA to the right, all in a pUC background. The site of deletion within vaccinia sequences is indicated by a triangle in FIG. 6. To avoid possible recombination of Beta-galactosidase sequences in the pUC-derived 35 portion of pSD539 with Beta-galactosidase sequences in recombinant vaccinia virus vP855, the vaccinia I4L deletion cassette was moved from pSD539 into pRC11, a pUC derivative from which all Beta-galactosidase sequences have been removed and replaced with a polylinker region 40 (Colinas et al., 1990). pSD539 was cut with EcoRI/PstI

and the 1.2 kb fragment isolated. This fragment was ligated into pRC11 cut with EcoRI/PstI (2.35 kb), forming pSD548. Recombination between pSD548 and the Beta-galactosidase containing vaccinia recombinant, vP855, 5 resulted in vaccinia deletion mutant vP866, which was isolated as a clear plaque in the presence of X-gal.

DNA from recombinant vaccinia virus vP866 was analyzed by restriction digests followed by electrophoresis on an agarose gel. The restriction 10 patterns were as expected. Polymerase chain reactions (PCR) (Engelke et al., 1988) using vP866 as template and primers flanking the six deletion loci detailed above produced DNA fragments of the expected sizes. Sequence analysis of the PCR generated fragments around the areas 15 of the deletion junctions confirmed that the junctions were as expected. Recombinant vaccinia virus vP866, containing the six engineered deletions as described above, was designated vaccinia vaccine strain "NYVAC."

Example 7 - INSERTION OF A RABIES GLYCOPROTEIN G GENE INTO NYVAC

The gene encoding rabies glycoprotein G under the control of the vaccinia H6 promoter (Taylor et al., 1988a,b) was inserted into TK deletion plasmid pSD513. 25 pSD513 is identical to plasmid pSD460 (FIG. 1) except for the presence of a polylinker region.

Referring now to FIG. 7, the polylinker region was inserted by cutting pSD460 with SmaI and ligating the plasmid vector with annealed synthetic oligonucleotides 30 VQ1A/VQ1B (SEQ ID NO:25/SEQ ID NO:26)

	<u>Sma</u> I	<u>Bgl</u> II	<u>Xho</u> I	<u>Pst</u> I	<u>Nar</u> I	<u>Bam</u> HI	
VQ1A	5'	GGGAGATCTCTCGAGCTGCAGGGCGCCGGATCCTTTTCT					3'
VQ1B	3'	CCCTCTAGAGAGCTCGACGTCCCGCGGCCTAGGAAAAAGA					5'

35 to form vector plasmid pSD513. pSD513 was cut with SmaI and ligated with a SmaI ended 1.8 kb cassette containing the gene encoding the rabies glycoprotein G gene under the control of the vaccinia H6 promoter (Taylor et al., 1988a,b). The resulting plasmid was designated pRW842. 40 pRW842 was used as donor plasmid for recombination with

NYVAC rescuing virus (vP866). Recombinant vaccinia virus vP879 was identified by plaque hybridization using ³²P-labelled DNA probe to rabies glycoprotein G coding sequences.

5 The modified recombinant viruses of the present invention provide advantages as recombinant vaccine vectors. The attenuated virulence of the vector advantageously reduces the opportunity for the possibility of a runaway infection due to vaccination in 10 the vaccinated individual and also diminishes transmission from vaccinated to unvaccinated individuals or contamination of the environment.

15 The modified recombinant viruses are also advantageously used in a method for expressing a gene product in a cell cultured *in vitro* by introducing into the cell the modified recombinant virus having foreign DNA which codes for and expresses gene products in the cell.

20 Example 8 - CONSTRUCTION OF TROVAC-NDV EXPRESSING THE FUSION AND HEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS

This example describes the development of TROVAC, a 25 fowlpox virus vector and, of a fowlpox Newcastle Disease Virus recombinant designated TROVAC-NDV and its safety and efficacy. A fowlpox virus (FPV) vector expressing both F and HN genes of the virulent NDV strain Texas was constructed. The recombinant produced was designated TROVAC-NDV. TROVAC-NDV expresses authentically processed 30 NDV glycoproteins in avian cells infected with the recombinant virus and inoculation of day old chicks protects against subsequent virulent NDV challenge.

35 Cells and Viruses. The Texas strain of NDV is a velogenic strain. Preparation of cDNA clones of the F and HN genes has been previously described (Taylor et al., 1990; Edbauer et al., 1990). The strain of FPV designated FP-1 has been described previously (Taylor et al., 1988a). It is a vaccine strain useful in vaccination of day old chickens. The parental virus

strain Duvette was obtained in France as a fowlpox scab from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chicken embryo fibroblast cells. The virus was subjected to four successive plaque purifications. One plaque isolate was further amplified in primary CEF cells and a stock virus, designated as TROVAC, established. The stock virus used in the *in vitro* recombination test to produce TROVAC-NDV had been subjected to twelve passages in primary CEF cells from the plaque isolate.

Construction of a Cassette for NDV-F. A 1.8 kbp BamHI fragment containing all but 22 nucleotides from the 5' end of the F protein coding sequence was excised from pNDV81 (Taylor et al., 1990) and inserted at the BamHI site of pUC18 to form pCE13. The vaccinia virus H6 promoter previously described (Taylor et al., 1988a,b; Guo et al., 1989; Perkus et al., 1989) was inserted into pCE13 by digesting pCE13 with SalI, filling in the sticky ends with Klenow fragment of *E. coli* DNA polymerase and digesting with HindIII. A HindIII - EcoRV fragment containing the H6 promoter sequence was then inserted into pCE13 to form pCE38. A perfect 5' end was generated by digesting pCE38 with KpnI and NruI and inserting the annealed and kinased oligonucleotides CE75 (SEQ ID NO:27) and CE76 (SEQ ID NO:28) to generate pCE47.

CE75:

CGATATCCGTTAAGTTGTATCGTAATGGGCTCCAGATCTTCTACCAGGATCCCGG
TAC

CE76:

CGGGATCCTGGTAGAAGATCTGGAGCCCATTACGATAACAACTTAACGGATATCG.

In order to remove non-coding sequence from the 3' end of the NDV-F a SmaI to PstI fragment from pCE13 was inserted into the SmaI and PstI sites of pUC18 to form pCE23. The non-coding sequences were removed by sequential digestion of pCE23 with SacI, BamHI, Exonuclease III, SI nuclease and EcoRI. The annealed and kinased oligonucleotides

CE42 (SEQ ID NO:29) and CE43 (SEQ ID NO:30) were then inserted to form pCE29.

CE42: AATTCGAGCTCCCCGGG

CE43: CCCGGGGAGCTCG

5 The 3' end of the NDV-F sequence was then inserted into plasmid pCE20 already containing the 5' end of NDV-F by cloning a PstI - SacI fragment from pCE29 into the PstI and SacI sites of pCE20 to form pCE32. Generation of pCE20 has previously been described in Taylor et al.,
10 1990.

In order to align the H6 promoter and NDV-F 5' sequences contained in pCE47 with the 3' NDV-F sequences contained in pCE32, a HindIII - PstI fragment of pCE47 was inserted into the HindIII and PstI sites of pCE32 to form pCE49. The H6 promoted NDV-F sequences were then transferred to the de-ORFed F8 locus (described below) by cloning a HindIII - NruI fragment from pCE49 into the HindIII and SmaI sites of pJCA002 (described below) to form pCE54. Transcription stop signals were inserted 15 into pCE54 by digesting pCE54 with SacI, partially digesting with BamHI and inserting the annealed and kinased oligonucleotides CE166 (SEQ ID NO:31) and CE167 (SEQ ID NO:32) to generate pCE58.

CE166: CTTTTATAAAAGTTAACTACGTAG

25 CE167: GATCCTACGTAGTTAACTTTATAAAAGAGCT

A perfect 3' end for NDV-F was obtained by using the polymerase chain reaction (PCR) with pCE54 as template and oligonucleotides CE182 (SEQ ID NO:33) and CE183 (SEQ ID NO:34) as primers.

30 CE182: CTTAACTCAGCTGACTATCC

CE183: TACGTAGTTAACTTTATAAAATCATATTTGTAGTGGCTC

The PCR fragment was digested with PvuII and HpaI and cloned into pCE58 that had been digested with HpaI and partially digested with PvuII. The resulting plasmid was 35 designated pCE64. Translation stop signals were inserted by cloning a HindIII - HpaI fragment which contains the complete H6 promoter and F coding sequence from pCE64

into the HindIII and HpaI sites of pRW846 to generate pCE71, the final cassette for NDV-F. Plasmid pRW846 is essentially equivalent to plasmid pJCA002 (described below) but containing the H6 promoter and transcription and translation stop signals. Digestion of pRW846 with HindIII and HpaI eliminates the H6 promoter but leaves the stop signals intact.

Construction of Cassette for NDV-HN. Construction of plasmid pRW802 was previously described in Edbauer et al., 1990. This plasmid contains the NDV-HN sequences linked to the 3' end of the vaccinia virus H6 promoter in a pUC9 vector. A HindIII - EcoRV fragment encompassing the 5' end of the vaccinia virus H6 promoter was inserted into the HindIII and EcoRV sites of pRW802 to form pRW830. A perfect 3' end for NDV-HN was obtained by inserting the annealed and kinased oligonucleotides CE162 (SEQ ID NO:35) and CE163 (SEQ ID NO:36) into the EcoRI site of pRW830 to form pCE59, the final cassette for NDV-HN.

CE162:

AATTCAAGGATCGTTCTTACTAGTTGAGATTCTCAAGGATGATGGGATTAAATT
TATAAGCTTG

CE163:

AATTCAAGCTTATAAAATTAAATCCCATCATCCTTGAGAATCTCAACTAGTAAAGG

AACGATCCTG

Construction of FPV Insertion Vector. Plasmid

pRW731-15 contains a 10kb PvuII - PvuII fragment cloned from genomic DNA. The nucleotide sequence was determined on both strands for a 3660 bp PvuII - EcoRV fragment.

The limits of an open reading frame designated here as F8 were determined. Plasmid pRW761 is a sub-clone of pRW731-15 containing a 2430 bp EcoRV - EcoRV fragment. The F8 ORF was entirely contained between an XbaI site and an SspI site in pRW761. In order to create an insertion plasmid which on recombination with TROVAC genomic DNA would eliminate the F8 ORF, the following steps were followed. Plasmid pRW761 was completely

digested with XbaI and partially digested with SspI. A 3700 bp XbaI - SspI band was isolated from the gel and ligated with the annealed double-stranded oligonucleotides JCA017 (SEQ ID NO:37) and JCA018 (SEQ ID NO:38).

5 JCA017:5'

CTAGACACTTATGTTTTAATATCCGGTCTAAAAGCTTCCGGGATCCTTATA

CGGGGAATAAT

JCA018:5'

10 ATTATTCCCCGTATAAGGATCCCCGGGAAGCTTTAAGACCGGATATTAAAAAACA
TAAAGTGT

The plasmid resulting from this ligation was designated pJCA002.

Construction of Double Insertion Vector for NDV F

15 and HN. The H6 promoted NDV-HN sequence was inserted into the H6 promoted NDV-F cassette by cloning a HindIII fragment from pCE59 that had been filled in with Klenow fragment of *E. coli* DNA polymerase into the HpaI site of pCE71 to form pCE80. Plasmid pCE80 was completely 20 digested with NdeI and partially digested with BglII to generate an NdeI - BglII 4760 bp fragment containing the NDV F and HN genes both driven by the H6 promoter and linked to F8 flanking arms. Plasmid pJCA021 was obtained by inserting a 4900 bp PvuII - HindII fragment from 25 pRW731-15 into the SmaI and HindII sites of pBSSK+.

Plasmid pJCA021 was then digested with NdeI and BglII and ligated to the 4760 bp NdeI - BglII fragment of pCE80 to form pJCA024. Plasmid pJCA024 therefore contains the NDV-F and HN genes inserted in opposite orientation with 30 3' ends adjacent between FPV flanking arms. Both genes are linked to the vaccinia virus H6 promoter. The right flanking arm adjacent to the NDV-F sequence consists of 2350 bp of FPV sequence. The left flanking arm adjacent to the NDV-HN sequence consists of 1700 bp of FPV 35 sequence.

Development of TROVAC-NDV. Plasmid pJCA024 was transfected into TROVAC infected primary CEF cells by

using the calcium phosphate precipitation method previously described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific NDV-F and HN radiolabelled probes and subjected to five sequential rounds of plaque purification until a pure population was achieved. One representative plaque was then amplified and the resulting TROVAC recombinant was designated TROVAC-NDV (vFP96).

10 Immunofluorescence. Indirect immunofluorescence was performed as described (Taylor et al., 1990) using a polyclonal anti-NDV serum and, as mono-specific reagents, sera produced in rabbits against vaccinia virus recombinants expressing NDV-F or NDV-HN.

15 Immunoprecipitation. Immunoprecipitation reactions were performed as described (Taylor et al., 1990) using a polyclonal anti-NDV serum obtained from SPAFAS Inc., Storrs, CT.

The stock virus was screened by *in situ* plaque 20 hybridization to confirm that the F8 ORF was deleted. The correct insertion of the NDV genes into the TROVAC genome and the deletion of the F8 ORF was also confirmed by Southern blot hybridization.

25 In NDV-infected cells, the F glycoprotein is anchored in the membrane via a hydrophobic transmembrane region near the carboxyl terminus and requires post-translational cleavage of a precursor, F₀, into two disulfide linked polypeptides F₁ and F₂. Cleavage of F₀ is important in determining the pathogenicity of a given 30 NDV strain (Homma and Ohuchi, 1973; Nagai et al., 1976; Nagai et al., 1980), and the sequence of amino acids at the cleavage site is therefore critical in determining viral virulence. It has been determined that amino acids at the cleavage site in the NDV-F sequence inserted into 35 FPV to form recombinant vFP29 had the sequence Arg - Arg - Gln - Arg - Arg (SEQ ID NO:39) (Taylor et al., 1990) which conforms to the sequence found to be a requirement

for virulent NDV strains (Chambers et al., 1986; Espion et al., 1987; Le et al., 1988; McGinnes and Morrison, 1986; Toyoda et al., 1987). The HN glycoprotein synthesized in cells infected with virulent strains of 5 NDV is an uncleaved glycoprotein of 74 kDa. Extremely avirulent strains such as Ulster and Queensland encode an HN precursor (HNo) which requires cleavage for activation (Garten et al., 1980).

10 The expression of F and HN genes in TROVAC-NDV was analyzed to confirm that the gene products were authentically processed and presented. Indirect-immunofluorescence using a polyclonal anti-NDV chicken serum confirmed that immunoreactive proteins were presented on the infected cell surface. To determine 15 that both proteins were presented on the plasma membrane, mono-specific rabbit sera were produced against vaccinia recombinants expressing either the F or HN glycoproteins. Indirect immunofluorescence using these sera confirmed the surface presentation of both proteins.

20 Immunoprecipitation experiments were performed by using (³⁵S) methionine labeled lysates of CEF cells infected with parental and recombinant viruses. The expected values of apparent molecular weights of the glycolysated forms of F₁ and F₂ are 54.7 and 10.3 kDa 25 respectively (Chambers et al., 1986). In the immunoprecipitation experiments using a polyclonal anti-NDV serum, fusion specific products of the appropriate size were detected from the NDV-F single recombinant vFP29 (Taylor et al., 1990) and the TROVAC-NDV double 30 recombinant vFP96. The HN glycoprotein of appropriate size was also detected from the NDV-HN single recombinant VFP-47 (Edbauer et al., 1990) and TROVAC-NDV. No NDV specific products were detected from uninfected and parental TROVAC infected CEF cells.

35 In CEF cells, the F and HN glycoproteins are appropriately presented on the infected cell surface where they are recognized by NDV immune serum.

Immunoprecipitation analysis indicated that the F_0 protein is authentically cleaved to the F_1 and F_2 components required in virulent strains. Similarly, the HN glycoprotein was authentically processed in CEF cells 5 infected with recombinant TROVAC-NDV.

Previous reports (Taylor et al., 1990; Edbauer et al., 1990; Boursnell et al., 1990a,b,c; Ogawa et al., 1990) would indicate that expression of either HN or F alone is sufficient to elicit protective immunity against 10 NDV challenge. Work on other paramyxoviruses has indicated, however, that antibody to both proteins may be required for full protective immunity. It has been demonstrated that SV5 virus could spread in tissue culture in the presence of antibody to the HN 15 glycoprotein but not to the F glycoprotein (Merz et al., 1980). In addition, it has been suggested that vaccine failures with killed measles virus vaccines were due to inactivation of the fusion component (Norrbjörn et al., 1975). Since both NDV glycoproteins have been shown to 20 be responsible for eliciting virus neutralizing antibody (Avery et al., 1979) and both glycoproteins, when expressed individually in a fowlpox vector are able to induce a protective immune response, it can be appreciated that the most efficacious NDV vaccine should 25 express both glycoproteins.

Example 9 - CONSTRUCTION OF ALVAC RECOMBINANTS
EXPRESSING RABIES VIRUS GLYCOPROTEIN G

This example describes the development of ALVAC, a 30 canarypox virus vector and, of a canarypox-rabies recombinant designated as ALVAC-RG (vCP65) and its safety and efficacy.

Cells and Viruses. The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. 35 The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under

agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in *in vitro* recombination tests. The plaque purified canarypox isolate is designated

5 ALVAC.

Construction of a Canarypox Insertion Vector. An 880 bp canarypox PvuII fragment was cloned between the PvuII sites of pUC9 to form pRW764.5. The sequence of this fragment is shown in FIG. 8 between positions 1372 and 2251. The limits of an open reading frame designated as C5 were defined. It was determined that the open reading frame was initiated at position 166 within the fragment and terminated at position 487. The C5 deletion was made without interruption of open reading frames.

10 Bases from position 167 through position 455 were replaced with the sequence (SEQ ID NO:39)

15 GCTTCCCGGGATTCTAGCTAGCTAGTTT. This replacement sequence contains HindIII, SmaI and EcoRI insertion sites followed by translation stops and a transcription termination

20 signal recognized by vaccinia virus RNA polymerase (Yuen et al., 1987). Deletion of the C5 ORF was performed as described below. Plasmid pRW764.5 was partially cut with RsaI and the linear product was isolated. The RsaI linear fragment was recut with BglII and the pRW764.5

25 fragment now with a RsaI to BglII deletion from position 156 to position 462 was isolated and used as a vector for the following synthetic oligonucleotides:

RW145 (SEQ ID NO:40):

ACTCTAAAAGCTTCCGGGAATTCTAGCTAGCTAGTTTATAAA

30 RW146 (SEQ ID NO:41):

GATCTTATAAAAATAGCTAGCTAGAATTCCGGGAAGCTTTGAGAGT

Oligonucleotides RW145 and RW146 were annealed and inserted into the pRW 764.5 RsaI and BglII vector described above. The resulting plasmid is designated

35 pRW831.

Construction of Insertion Vector Containing the Rabies G Gene. Construction of pRW838 is illustrated

below. Oligonucleotides A through E, which overlap the translation initiation codon of the H6 promoter with the ATG of rabies G, were cloned into pUC9 as PRW737. Oligonucleotides A through E contain the H6 promoter, starting at NruI, through the HindIII site of rabies G followed by BglIII. Sequences of oligonucleotides A through E ((SEQ ID NO:42)-(SEQ ID NO:46)) are:

A (SEQ ID NO:42): CTGAAATTATTCATTATCGCGATATCCGTTAAGTTGTATCGTAATGGTTCCCTCAGGCTCTCCTGTTGT

10 B (SEQ ID NO:43): CATTACGATACAAACTTAACGGATATCGCGATAA
TGAAATAATTCAG

C (SEQ ID NO:44): ACCCCCTCTGGTTTTCCGTTGTGTTGGGAA
TTCCCTATTTACACGATCCCAGACAAGCTTAGATCTCAG

D (SEQ ID NO:45): CTGAGATCTAAGCTTGTCTGGGATCGTGTAAATA
GGGAATTCCTCCAAAACA

15 GGGAAATTCCCCAAACCA

E (SEQ ID NO:46): CAACGGAAAAACCAAGGGGGTACAAAACAGGGAGA
GCCTGAGGAAC

The diagram of annealed oligonucleotides A through E is as follows:

Figure 1 consists of two horizontal dotted lines representing a scale. The top line has a vertical tick mark at point A. The bottom line has tick marks at points B, E, and D. The segment between B and E is labeled with a length of 20. The segment between E and D is also labeled with a length of 20.

Oligonucleotides A through E were kinased, annealed (95°C for 5 minutes, then cooled to room temperature), and inserted between the PvuII sites of pUC9. The resulting plasmid, pRW737, was cut with HindIII and BglII and used as a vector for the 1.6 kbp HindIII-BglII fragment of ptg155PRO (Kieny et al., 1984) generating pRW739. The ptg155PRO HindIII site is 86 bp downstream of the rabies G translation initiation codon. BglII is downstream of the rabies G translation stop codon in ptg155PRO. pRW739 was partially cut with NruI, completely cut with BglII, and a 1.7 kbp NruI-BglII fragment, containing the 3' end of the H6 promoter previously described (Taylor et al., 1988a,b; Guo et al.,

1989; Perkus et al., 1989) through the entire rabies G gene, was inserted between the NruI and BamHI sites of pRW824. The resulting plasmid is designated pRW832. Insertion into pRW824 added the H6 promoter 5' of NruI. 5 The pRW824 sequence of BamHI followed by SmaI is (SEQ ID NO:47): GGATCCCCGGG. pRW824 is a plasmid that contains a nonpertinent gene linked precisely to the vaccinia virus H6 promoter. Digestion with NruI and BamHI completely excised this nonpertinent gene. The 1.8 kbp pRW832 SmaI 10 fragment, containing H6 promoted rabies G, was inserted into the SmaI of pRW831, to form plasmid pRW838.

Development of ALVAC-RG. Plasmid pRW838 was transfected into ALVAC infected primary CEF cells by using the calcium phosphate precipitation method 15 previously described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to a specific rabies G probe and subjected to 6 sequential rounds of plaque purification until a pure population was achieved. One representative 20 plaque was then amplified and the resulting ALVAC recombinant was designated ALVAC-RG (vCP65) (see also FIG. 9). The correct insertion of the rabies G gene into the ALVAC genome without subsequent mutation was confirmed by sequence analysis.

25 Immunofluorescence. During the final stages of assembly of mature rabies virus particles, the glycoprotein component is transported from the golgi apparatus to the plasma membrane where it accumulates with the carboxy terminus extending into the cytoplasm 30 and the bulk of the protein on the external surface of the cell membrane. In order to confirm that the rabies glycoprotein expressed in ALVAC-RG was correctly presented, immunofluorescence was performed on primary CEF cells infected with ALVAC or ALVAC-RG. 35 Immunofluorescence was performed as previously described (Taylor et al., 1990) using a rabies G monoclonal antibody. Strong surface fluorescence was detected on

CEF cells infected with ALVAC-RG but not with the parental ALVAC.

5 Immunoprecipitation. Preformed monolayers of primary CEF, Vero (a line of African Green monkey kidney cells ATCC # CCL81) and MRC-5 cells (a fibroblast-like cell line derived from normal human fetal lung tissue ATCC # CCL171) were inoculated at 10 pfu per cell with parental virus ALVAC and recombinant virus ALVAC-RG in the presence of radiolabelled ^{35}S -methionine and treated 10 as previously described (Taylor et al., 1990).

15 Immunoprecipitation reactions were performed using a rabies G specific monoclonal antibody. Efficient expression of a rabies specific glycoprotein with a molecular weight of approximately 67 kDa was detected with the recombinant ALVAC-RG. No rabies specific 20 products were detected in uninfected cells or cells infected with the parental ALVAC virus.

Sequential Passaging Experiment. In studies with 25 ALVAC virus in a range of non-avian species no proliferative infection or overt disease was observed (Taylor et al., 1991b). However, in order to establish that neither the parental nor recombinant virus could be adapted to grow in non-avian cells, a sequential 30 passaging experiment was performed.

The two viruses, ALVAC and ALVAC-RG, were inoculated 35 in 10 sequential blind passages in three cell lines:

- (1) Primary chick embryo fibroblast (CEF) cells produced from 11 day old white leghorn embryos;
- (2) Vero cells - a continuous line of African Green 40 monkey kidney cells (ATCC # CCL81); and
- (3) MRC-5 cells - a diploid cell line derived from human fetal lung tissue (ATCC # CCL171).

The initial inoculation was performed at an m.o.i. of 0.1 pfu per cell using three 60mm dishes of each cell line 45 containing 2×10^6 cells per dish. One dish was inoculated in the presence of 40 $\mu\text{g}/\text{ml}$ of Cytosine arabinoside (Ara C), an inhibitor of DNA replication.

After an absorption period of 1 hour at 37°C, the inoculum was removed and the monolayer washed to remove unabsorbed virus. At this time the medium was replaced with 5ml of EMEM + 2% NBCS on two dishes (samples t0 and 5 t7) and 5ml of EMEM + 2% NBCS containing 40 µg/ml Ara C on the third (sample t7A). Sample t0 was frozen at -70°C to provide an indication of the residual input virus. Samples t7 and t7A were incubated at 37°C for 7 days, after which time the contents were harvested and the 10 cells disrupted by indirect sonication.

One ml of sample t7 of each cell line was inoculated undiluted onto three dishes of the same cell line (to provide samples t0, t7 and t7A) and onto one dish of primary CEF cells. Samples t0, t7 and t7A were treated 15 as for passage one. The additional inoculation on CEF cells was included to provide an amplification step for more sensitive detection of virus which might be present in the non-avian cells.

This procedure was repeated for 10 (CEF and MRC-5) 20 or 8 (Vero) sequential blind passages. Samples were then frozen and thawed three times and assayed by titration on primary CEF monolayers.

Virus yield in each sample was then determined by 25 plaque titration on CEF monolayers under agarose. Summarized results of the experiment are shown in Tables 1 and 2.

The results indicate that both the parental ALVAC and the recombinant ALVAC-RG are capable of sustained replication on CEF monolayers with no loss of titer. In 30 Vero cells, levels of virus fell below the level of detection after 2 passages for ALVAC and 1 passage for ALVAC-RG. In MRC-5 cells, a similar result was evident, and no virus was detected after 1 passage. Although the results for only four passages are shown in Tables 1 and 35 2 the series was continued for 8 (Vero) and 10 (MRC-5) passages with no detectable adaptation of either virus to growth in the non-avian cells.

In passage 1 relatively high levels of virus were present in the t7 sample in MRC-5 and Vero cells. However this level of virus was equivalent to that seen in the t0 sample and the t7A sample incubated in the 5 presence of Cytosine arabinoside in which no viral replication can occur. This demonstrated that the levels of virus seen at 7 days in non-avian cells represented residual virus and not newly replicated virus.

In order to make the assay more sensitive, a portion 10 of the 7 day harvest from each cell line was inoculated onto a permissive CEF monolayer and harvested at cytopathic effect (CPE) or at 7 days if no CPE was evident. The results of this experiment are shown in Table 3. Even after amplification through a permissive 15 cell line, virus was only detected in MRC-5 and Vero cells for two additional passages. These results indicated that under the conditions used, there was no adaptation of either virus to growth in Vero or MRC-5 cells.

20 Inoculation of Macaques. Four HIV seropositive macaques were initially inoculated with ALVAC-RG as described in Table 4. After 100 days these animals were re-inoculated to determine a booster effect, and an additional seven animals were inoculated with a range of 25 doses. Blood was drawn at appropriate intervals and sera analyzed, after heat inactivation at 56°C for 30 minutes, for the presence of anti-rabies antibody using the Rapid Fluorescent Focus Inhibition Assay (Smith et al., 1973).

Inoculation of Chimpanzees. Two adult male 30 chimpanzees (50 to 65 kg weight range) were inoculated intramuscularly or subcutaneously with 1×10^7 pfu of vCP65. Animals were monitored for reactions and bled at regular intervals for analysis for the presence of anti-rabies antibody with the RFFI test (Smith et al., 1973). 35 Animals were re-inoculated with an equivalent dose 13 weeks after the initial inoculation.

Inoculation of Mice. Groups of mice were inoculated with 50 to 100 μ l of a range of dilutions of different batches of vCP65. Mice were inoculated in the footpad. On day 14, mice were challenged by intracranial 5 inoculation of from 15 to 43 mouse LD₅₀ of the virulent CVS strain of rabies virus. Survival of mice was monitored and a protective dose 50% (PD₅₀) calculated at 28 days post-inoculation.

Inoculation of Dogs and Cats. Ten beagle dogs, 10 months old, and 10 cats, 4 months old, were inoculated subcutaneously with either 6.7 or 7.7 log₁₀ TCID₅₀ of ALVAC-RG. Four dogs and four cats were not inoculated. Animals were bled at 14 and 28 days post-inoculation and anti-rabies antibody assessed in an RFFI test. The 15 animals receiving 6.7 log₁₀ TCID₅₀ of ALVAC-RG were challenged at 29 days post-vaccination with 3.7 log₁₀ mouse LD₅₀ (dogs) or 4.3 log₁₀ mouse LD₅₀ (cats) of the NYGS rabies virus challenge strain.

Inoculation of Squirrel Monkeys. Three groups of 20 four squirrel monkeys (*Saimiri sciureus*) were inoculated with one of three viruses (a) ALVAC, the parental canarypox virus, (b) ALVAC-RG, the recombinant expressing the rabies G glycoprotein or (c) vCP37, a canarypox recombinant expressing the envelope glycoprotein of 25 feline leukemia virus. Inoculations were performed under ketamine anaesthesia. Each animal received at the same time: (1) 20 μ l instilled on the surface of the right eye without scarification; (2) 100 μ l as several droplets in the mouth; (3) 100 μ l in each of two intradermal 30 injection sites in the shaven skin of the external face of the right arm; and (4) 100 μ l in the anterior muscle of the right thigh.

Four monkeys were inoculated with each virus, two with a total of 5.0 log₁₀ pfu and two with a total of 7.0 35 log₁₀ pfu. Animals were bled at regular intervals and sera analyzed for the presence of antirabies antibody using an RFFI test (Smith et al., 1973). Animals were

monitored daily for reactions to vaccination. Six months after the initial inoculation the four monkeys receiving ALVAC-RG, two monkeys initially receiving vCP37, and two monkeys initially receiving ALVAC, as well as one naive 5 monkey were inoculated with $6.5 \log_{10}$ pfu of ALVAC-RG subcutaneously. Sera were monitored for the presence of rabies neutralizing antibody in an RFFI test (Smith et al., 1973).

Inoculation of Human Cell Lines with ALVAC-RG. In 10 order to determine whether efficient expression of a foreign gene could be obtained in non-avian cells in which the virus does not productively replicate, five cell types, one avian and four non-avian, were analyzed for virus yield, expression of the foreign rabies G gene 15 and viral specific DNA accumulation. The cells inoculated were:

- (a) Vero, African Green monkey kidney cells, ATCC # CCL81;
- (b) MRC-5, human embryonic lung, ATCC # CCL 171;
- (c) WISH human amnion, ATCC # CCL 25;
- (d) Detroit-532, human foreskin, Downs's syndrome, ATCC # CCL 54; and
- (e) Primary CEF cells.

20 Chicken embryo fibroblast cells produced from 11 day old white leghorn embryos were included as a positive 25 control. All inoculations were performed on preformed monolayers of 2×10^6 cells as discussed below.

A. Methods for DNA analysis.

30 Three dishes of each cell line were inoculated at 5 pfu/cell of the virus under test, allowing one extra dish of each cell line un-inoculated. One dish was 35 incubated in the presence of 40 $\mu\text{g}/\text{ml}$ of cytosine arabinoside (Ara C). After an adsorption period of 60 minutes at 37°C, the inoculum was removed and the monolayer washed twice to remove unadsorbed virus. Medium (with or without Ara C) was then replaced. Cells from one dish (without Ara C) were harvested

as a time zero sample. The remaining dishes were incubated at 37°C for 72 hours, at which time the cells were harvested and used to analyze DNA accumulation. Each sample of 2×10^6 cells was resuspended in 0.5 ml phosphate buffered saline (PBS) containing 40 mM EDTA and incubated for 5 minutes at 37°C. An equal volume of 1.5% agarose prewarmed at 42°C and containing 120 mM EDTA was added to the cell suspension and gently mixed. The suspension was transferred to an agarose plug mold and allowed to harden for at least 15 min. The agarose plugs were then removed and incubated for 12-16 hours at 50°C in a volume of lysis buffer (1% sarkosyl, 100 µg/ml proteinase K, 10 mM Tris HCl pH 7.5, 200 mM EDTA) that completely covers the plug. The lysis buffer was then replaced with 5.0 ml sterile 0.5 X TBE (44.5 mM Tris-borate, 44.5 mM boric acid, 0.5 mM EDTA) and equilibrated at 4°C for 6 hours with 3 changes of TBE buffer. The viral DNA within the plug was fractionated from cellular RNA and DNA using a pulse field electrophoresis system. Electrophoresis was performed for 20 hours at 180 V with a ramp of 50-90 sec at 15°C in 0.5 X TBE. The DNA was run with lambda DNA molecular weight standards. After electrophoresis the viral DNA band was visualized by staining with ethidium bromide. The DNA was then transferred to a nitrocellulose membrane and probed with a radiolabelled probe prepared from purified ALVAC genomic DNA.

30 B. Estimation of virus yield.

Dishes were inoculated exactly as described above, with the exception that input multiplicity was 0.1 pfu/cell. At 72 hours post infection, cells were lysed by three successive cycles of freezing and thawing. Virus yield was assessed by plaque titration on CEF monolayers.

C. Analysis of expression of Rabies G gene.

Dishes were inoculated with recombinant or parental virus at a multiplicity of 10 pfu/cell, allowing an additional dish as an uninfected virus control.

5 After a one hour absorption period, the medium was removed and replaced with methionine free medium.

After a 30 minute period, this medium was replaced with methionine-free medium containing 25 uCi/ml of ³⁵S-Methionine. Infected cells were labelled 10 overnight (approximately 16 hours), then lysed by the addition of buffer A lysis buffer.

Immunoprecipitation was performed as previously described (Taylor et al., 1990) using a rabies G specific monoclonal antibody.

15 Results: Estimation of Viral Yield. The results of titration for yield at 72 hours after inoculation at 0.1 pfu per cell are shown in Table 5. The results indicate that while a productive infection can be attained in the avian cells, no increase in virus yield can be detected 20 by this method in the four non-avian cell systems.

Analysis of Viral DNA Accumulation. In order to determine whether the block to productive viral replication in the non-avian cells occurred before or after DNA replication, DNA from the cell lysates was 25 fractionated by electrophoresis, transferred to nitrocellulose and probed for the presence of viral specific DNA. DNA from uninfected CEF cells, ALVAC-RG infected CEF cells at time zero, ALVAC-RG infected CEF cells at 72 hours post-infection and ALVAC-RG infected 30 CEF cells at 72 hours post-infection in the presence of 40 µg/ml of cytosine arabinoside all showed some background activity, probably due to contaminating CEF cellular DNA in the radiolabelled ALVAC DNA probe preparation. However, ALVAC-RG infected CEF cells at 72 35 hours post-infection exhibited a strong band in the region of approximately 350 kbp representing ALVAC-specific viral DNA accumulation. No such band is

detectable when the culture is incubated in the presence of the DNA synthesis inhibitor, cytosine arabinoside. Equivalent samples produced in Vero cells showed a very faint band at approximately 350 kbp in the ALVAC-RG 5 infected Vero cells at time zero. This level represented residual virus. The intensity of the band was amplified at 72 hours post-infection indicating that some level of viral specific DNA replication had occurred in Vero cells which had not resulted in an increase in viral progeny. 10 Equivalent samples produced in MRC-5 cells indicated that no viral specific DNA accumulation was detected under these conditions in this cell line. This experiment was then extended to include additional human cell lines, specifically WISH and Detroit-532 cells. ALVAC infected 15 CEF cells served as a positive control. No viral specific DNA accumulation was detected in either WISH or Detroit cells inoculated with ALVAC-RG. It should be noted that the limits of detection of this method have not been fully ascertained and viral DNA accumulation may 20 be occurring, but at a level below the sensitivity of the method. Other experiments in which viral DNA replication was measured by ³H-thymidine incorporation support the results obtained with Vero and MRC-5 cells.

Analysis of Rabies Gene Expression. To determine if 25 any viral gene expression, particularly that of the inserted foreign gene, was occurring in the human cell lines even in the absence of viral DNA replication, immunoprecipitation experiments were performed on ³⁵S-methionine labelled lysates of avian and non-avian cells 30 infected with ALVAC and ALVAC-RG. The results of immunoprecipitation using a rabies G specific monoclonal antibody illustrated specific immunoprecipitation of a 67 kDa glycoprotein in CEF, Vero and MRC-5, WISH and Detroit cells infected with ALVAC-RG. No such specific rabies 35 gene products were detected in any of the uninfected and parentally infected cell lysates.

The results of this experiment indicated that in the human cell lines analyzed, although the ALVAC-RG recombinant was able to initiate an infection and express a foreign gene product under the transcriptional control 5 of the H6 early/late vaccinia virus promoter, the replication did not proceed through DNA replication, nor was there any detectable viral progeny produced. In the Vero cells, although some level of ALVAC-RG specific DNA accumulation was observed, no viral progeny was detected 10 by these methods. These results would indicate that in the human cell lines analyzed the block to viral replication occurs prior to the onset of DNA replication, while in Vero cells, the block occurs following the onset 15 of viral DNA replication.

15 In order to determine whether the rabies glycoprotein expressed in ALVAC-RG was immunogenic, a number of animal species were tested by inoculation of the recombinant. The efficacy of current rabies vaccines is evaluated in a mouse model system. A similar test was 20 therefore performed using ALVAC-RG. Nine different preparations of virus (including one vaccine batch (J) produced after 10 serial tissue culture passages of the seed virus) with infectious titers ranging from 6.7 to 25 8.4 \log_{10} TCID₅₀ per ml were serially diluted and 50 to 100 μ l of dilutions inoculated into the footpad of four to six week old mice. Mice were challenged 14 days later by the intracranial route with 300 μ l of the CVS strain 30 of rabies virus containing from 15 to 43 mouse LD₅₀ as determined by lethality titration in a control group of mice. Potency, expressed as the PD₅₀ (Protective dose 50%), was calculated at 14 days post-challenge. The results of the experiment are shown in Table 6. The results indicated that ALVAC-RG was consistently able to 35 protect mice against rabies virus challenge with a PD₅₀ value ranging from 3.33 to 4.56 with a mean value of 3.73 (STD 0.48). As an extension of this study, male mice were inoculated intracranially with 50 μ l of virus

containing $6.0 \log_{10}$ TCID₅₀ of ALVAC-RG or with an equivalent volume of an uninfected cell suspension. Mice were sacrificed on days 1, 3 and 6 post-inoculation and their brains removed, fixed and sectioned.

5 Histopathological examination showed no evidence for neurovirulence of ALVAC-RG in mice.

In order to evaluate the safety and efficacy of ALVAC-RG for dogs and cats, a group of 14, 5 month old beagles and 14, 4 month old cats were analyzed. Four 10 animals in each species were not vaccinated. Five animals received $6.7 \log_{10}$ TCID₅₀ subcutaneously and five animals received $7.7 \log_{10}$ TCID₅₀ by the same route. Animals were bled for analysis for anti-rabies antibody. Animals receiving no inoculation or $6.7 \log_{10}$ TCID₅₀ of 15 ALVAC-RG were challenged at 29 days post-vaccination with $3.7 \log_{10}$ mouse LD₅₀ (dogs, in the temporal muscle) or $4.3 \log_{10}$ mouse LD₅₀ (cats, in the neck) of the NYGS rabies virus challenge strain. The results of the experiment are shown in Table 7.

20 No adverse reactions to inoculation were seen in either cats or dogs with either dose of inoculum virus. Four of 5 dogs immunized with $6.7 \log_{10}$ TCID₅₀ had antibody titers on day 14 post-vaccination and all dogs had titers at 29 days. All dogs were protected from a 25 challenge which killed three out of four controls. In cats, three of five cats receiving $6.7 \log_{10}$ TCID₅₀ had specific antibody titers on day 14 and all cats were positive on day 29 although the mean antibody titer was low at 2.9 IU. Three of five cats survived a challenge 30 which killed all controls. All cats immunized with $7.7 \log_{10}$ TCID₅₀ had antibody titers on day 14 and at day 29 the Geometric Mean Titer was calculated as 8.1 International Units.

The immune response of squirrel monkeys (*Saimiri sciureus*) to inoculation with ALVAC, ALVAC-RG and an unrelated canarypox virus recombinant was examined. Groups of monkeys were inoculated as described above and

sera analyzed for the presence of rabies specific antibody. Apart from minor typical skin reactions to inoculation by the intradermal route, no adverse reactivity was seen in any of the monkeys. Small amounts of residual virus were isolated from skin lesions after intradermal inoculation on days two and four post-inoculation only. All specimens were negative on day seven and later. There was no local reaction to intramuscular injection. All four monkeys inoculated with ALVAC-RG developed anti-rabies serum neutralizing antibodies as measured in an RFFI test. Approximately six months after the initial inoculation all monkeys and one additional naive monkey were re-inoculated by the subcutaneous route on the external face of the left thigh with $6.5 \log_{10}$ TCID₅₀ of ALVAC-RG. Sera were analyzed for the presence of anti-rabies antibody. The results are shown in Table 8.

Four of the five monkeys naive to rabies developed a serological response by seven days post-inoculation with ALVAC-RG. All five monkeys had detectable antibody by 11 days post-inoculation. Of the four monkeys with previous exposure to the rabies glycoprotein, all showed a significant increase in serum neutralization titer between days 3 and 7 post-vaccination. The results indicate that vaccination of squirrel monkeys with ALVAC-RG does not produce adverse side-effects and a primary neutralizing antibody response can be induced. An amnanestic response is also induced on re-vaccination. Prior exposure to ALVAC or to a canarypox recombinant expressing an unrelated foreign gene does not interfere with induction of an anti-rabies immune response upon re-vaccination.

The immunological response of HIV-2 seropositive macaques to inoculation with ALVAC-RG was assessed. Animals were inoculated as described above and the presence of anti-rabies serum neutralizing antibody assessed in an RFFI test. The results, shown in Table 9,

indicated that HIV-2 positive animals inoculated by the subcutaneous route developed anti-rabies antibody by 11 days after one inoculation. An anamnestic response was detected after a booster inoculation given approximately 5 three months after the first inoculation. No response was detected in animals receiving the recombinant by the oral route. In addition, a series of six animals were inoculated with decreasing doses of ALVAC-RG given by either the intra-muscular or subcutaneous routes. Five 10 of the six animals inoculated responded by 14 days post-vaccination with no significant difference in antibody titer.

Two chimpanzees with prior exposure to HIV were inoculated with $7.0 \log_{10}$ pfu of ALVAC-RG by the 15 subcutaneous or intra-muscular route. At 3 months post-inoculations both animals were re-vaccinated in an identical fashion. The results are shown in Table 10.

No adverse reactivity to inoculation was noted by either intramuscular or subcutaneous routes. Both 20 chimpanzees responded to primary inoculation by 14 days and a strongly rising response was detected following re-vaccination.

Table 1. Sequential Passage of ALVAC in Avian and non-Avian Cells.

		<u>CEF</u>	<u>Vero</u>	<u>MRC-5</u>
5	Pass 1			
	Sample to ^a	2.4	3.0	2.6
	t7 ^b	7.0	1.4	0.4
	t7A ^c	1.2	1.2	0.4
10	Pass 2			
	Sample to	5.0	0.4	N.D. ^d
	t7	7.3	0.4	N.D.
	t7A	3.9	N.D.	N.D.
15	Pass 3			
	Sample to	5.4	0.4	N.D.
	t7	7.4	N.D.	N.D.
	t7A	3.8	N.D.	N.D.
20	Pass 4			
	Sample to	5.2	N.D.	N.D.
	t7	7.1	N.D.	N.D.
	t7A	3.9	N.D.	N.D.

25 a: This sample was harvested at zero time and represents the residual input virus. The titer is expressed as \log_{10} pfu per ml.

b: This sample was harvested at 7 days post-infection.

30 c: This sample was inoculated in the presence of 40 μ g/ml of Cytosine arabinoside and harvested at 7 days post infection.

d: Not detectable

Table 2. Sequential Passage of ALVAC-RG in Avian and non-Avian Cells

		CEF	Vero	MRC-5
5	Pass 1			
	Sample t0 ^a	3.0	2.9	2.9
	t7 ^b	7.1	1.0	1.4
	t7A ^c	1.8	1.4	1.2
10	Pass 2			
	Sample t0	5.1	0.4	0.4
	t7	7.1	N.D. ^d	N.D.
	t7A	3.8	N.D.	N.D.
15	Pass 3			
	Sample t0	5.1	0.4	N.D.
	t7	7.2	N.D.	N.D.
	t7A	3.6	N.D.	N.D.
20	Pass 4			
	Sample t0	5.1	N.D.	N.D.
	t7	7.0	N.D.	N.D.
	t7A	4.0	N.D.	N.D.

a: This sample was harvested at zero time and represents the residual input virus. The titer is expressed as \log_{10} pfu per ml.

b: This sample was harvested at 7 days post-infection.

c: This sample was inoculated in the presence of 40 μ g/ml of Cytosine arabinoside and harvested at 7 days post-infection.

d: Not detectable.

Table 3. Amplification of residual virus by passage in CEF cells

CEF	Vero	MRC-5	
5 a) ALVAC			
Pass 2 ^a	7.0 ^b	6.0	5.2
3	7.5	4.1	4.9
4	7.5	N.D. ^c	N.D.
5	7.1	N.D.	N.D.
10			
b) ALVAC-RG			
Pass 2 ^a	7.2	5.5	5.5
3	7.2	5.0	5.1
4	7.2	N.D.	N.D.
15 5	7.2	N.D.	N.D.

a: Pass 2 represents the amplification in CEF cells of the 7 day sample from Pass 1.

20 b: Titer expressed as \log_{10} pfu per ml

c: Not Detectable

Table 4. Schedule of inoculation of rhesus macaques with ALVAC-RG (vCP65)

5	Animal	Inoculation
	176L	Primary: 1×10^8 pfu of vCP65 orally in TANG Secondary: 1×10^7 pfu of vCP65 plus 1×10^7 pfu of vCP82 ^a by SC route
10	185 L	Primary: 1×10^8 pfu of vCP65 orally in Tang Secondary: 1×10^7 pfu of vCP65 plus 1×10^7 pfu of vCP82 by SC route
15	177 L	Primary: 5×10^7 pfu SC of vCP65 by SC route Secondary: 1×10^7 pfu of vCP65 plus 1×10^7 pfu of vCP82 by SC route
186L		Primary: 5×10^7 pfu of vCP65 by SC route Secondary: 1×10^7 pfu of vCP65 plus 1×10^7 pfu of vCP82 by SC route
178L		Primary: 1×10^7 pfu of vCP65 by SC route
20	182L	Primary: 1×10^7 pfu of vCP65 by IM route
	179L	Primary: 1×10^6 pfu of vCP65 by SC route
	183L	Primary: 1×10^6 pfu of vCP65 by IM route
	180L	Primary: 1×10^6 pfu of vCP65 by SC route
	184L	Primary: 1×10^5 pfu of vCP65 by IM route
25	187L	Primary 1×10^7 pfu of vCP65 orally

a: vCP82 is a canarypox virus recombinant expressing the measles virus fusion and hemagglutinin genes.

Table 5: Analysis of yield in avian and non-avian cells inoculated with ALVAC-RG

Sample Time		t0	t72	t72A ^b
5	Cell Type			
<hr/>				
Expt 1				
	CEF	3.3 ^a	7.4	1.7
	Vero	3.0	1.4	1.7
10	MRC-5	3.4	2.0	1.7
Expt 2				
	CEF	2.9	7.5	<1.7
	WISH	3.3	2.2	2.0
	Detroit-532	2.8	1.7	<1.7

15

a: Titer expressed as \log_{10} pfu per mlb: Culture incubated in the presence of 40 μ g/ml of
Cytosine arabinoside

Table 6. Potency of ALVAC-RG as tested in mice

	Test	Challenge Dose ^a	PD ₅₀ ^b
5	Initial seed	43	4.56
	Primary seed	23	3.34
	Vaccine Batch H	23	4.52
	Vaccine Batch I	23	3.33
	Vaccine Batch K	15	3.64
10	Vaccine Batch L	15	4.03
	Vaccine Batch M	15	3.32
	Vaccine Batch N	15	3.39
	Vaccine Batch J	23	3.42

15 a: Expressed as mouse LD₅₀

b: Expressed as log₁₀ TCID₅₀

Table 7: Efficacy of ALVAC-RG in dogs and cats

Dose	<u>Dogs</u>		<u>Cats</u>	
	Antibody ^a	Survival ^b	Antibody	Survival
6.7	11.9	5/5	2.9	3/5
7.7	10.1	N.T.	8.1	N.T.

a: Antibody at day 29 post inoculation expressed as the
geometric mean titer in International Units.

b: Expressed as a ratio of survivors over animals
challenged

Table 8. Anti-rabies serological response of Squirrel monkeys inoculated with canarypox recombinants

5	Monkey #	Previous Exposure	Rabies serum-neutralizing antibody ^a						
			-196 ^b	0	3	7	11	21	28
	22	ALVAC ^c	NT ^g	<1.2	<1.2	<1.2	2.1	2.3	2.2
	51	ALVAC ^c	NT	<1.2	<1.2	1.7	2.2	2.2	2.2
	39	vCP37 ^d	NT	<1.2	<1.2	1.7	2.1	2.2	N.T. ^g
10	55	vCP37 ^d	NT	<1.2	<1.2	1.7	2.2	2.1	N.T.
	37	ALVAC-RG ^e	2.2	<1.2	<1.2	3.2	3.5	3.5	3.2
	53	ALVAC-RG ^e	2.2	<1.2	<1.2	3.6	3.6	3.6	3.4
	38	ALVAC-RG ^f	2.7	<1.7	<1.7	3.2	3.8	3.6	N.T.
	54	ALVAC-RG ^f	3.2	<1.7	<1.5	3.6	4.2	4.0	3.6
15	57	None	NT	<1.2	<1.2	1.7	2.7	2.7	2.3

a: As determined by RFFI test on days indicated and expressed in International Units

b: Day-196 represents serum from day 28 after primary vaccination

c: Animals received 5.0 \log_{10} TCID₅₀ of ALVAC

d: Animals received 5.0 \log_{10} TCID₅₀ of vCP37

e: Animals received 5.0 \log_{10} TCID₅₀ of ALVAC-RG

f: Animals received 7.0 \log_{10} TCID₅₀ of ALVAC-RG

25 g: Not tested.

Table 9. Inoculation of rhesus macaques with ALVAC-RG^a

Days post- inocu- lation	Route of Primary Inoculation									
	or/Tang 176L ^b 185L	SC 177L	SC 186L	SC 178L	IM 182L	SC 179L	IM 183L	SC 180L	IM 184L	OR 187L ^c
10										
-84
-9
15	3
6	.	.	±	±
11	.	.	16 ^d	128
19	.	.	32	128
35	.	.	32	512
20	59	.	.	64	256
75	.	.	54	128
99 ^c	.	.	64	256
2	.	.	32	256
6	.	.	512	512
25	15	16	16	512	512	64	32	64	128	32
29	16	32	256	256	64	64	32	128	32	.
55		32				32		32	16	.
57	16		128	128	16		16			.
30										

a: See Table 9 for schedule of inoculations.

b: Animals 176L and 185L received 8.0 \log_{10} pfu by the
35 oral route in 5 ml Tang. Animal 187L received 7.0
 \log_{10} pfu by oral route not in Tang.

c: Day of re-vaccination for animals 176L, 185L, 177L
40 and 186L by S.C. route, and primary vaccination for
animals 178L, 182L, 179L, 183L, 180L, 184L and
187L.

d: Titters expressed as reciprocal of last dilution
showing inhibition of fluorescence in an RFFI test.

Table 10. Inoculation of chimpanzees with ALVAC-RG

Weeks post- Inoculation	Animal 431 I.M.	Animal 457 S.C.
5		
0	<8 ^a	<8
1	<8	<8
2	8	32
4	16	32
10	8	32
	12 ^b /0	8
	13/1	128
	15/3	256
	20/8	64
15	26/12	128

a: Titer expressed as reciprocal of last dilution
showing inhibition of fluorescence in an RFFI test

b: Day of re-inoculation

Example 10 - IMMUNIZATION OF HUMANS USING CANARYPOX
EXPRESSING RABIES GLYCOPROTEIN
(ALVAC-RG; vCP65)

5 ALVAC-RG (vCP65) was generated as described in
Example 9 and FIGS. 9A and 9B. For scaling-up and
vaccine manufacturing ALVAC-RG (vCP65) was grown in
primary CEF derived from specified pathogen free eggs.
Cells were infected at a multiplicity of 0.01 and
10 incubated at 37°C for three days.

15 The vaccine virus suspension was obtained by
ultrasonic disruption in serum free medium of the
infected cells; cell debris were then removed by
centrifugation and filtration. The resulting clarified
suspension was supplemented with lyophilization
stabilizer (mixture of amino-acids), dispensed in single
dose vials and freeze dried. Three batches of decreasing
titer were prepared by ten-fold serial dilutions of the
virus suspension in a mixture of serum free medium and
20 lyophilization stabilizer, prior to lyophilization.

25 Quality control tests were applied to the cell
substrates, media and virus seeds and final product with
emphasis on the search for adventitious agents and
innocuity in laboratory rodents. No undesirable trait
was found.

30 Preclinical data. Studies *in vitro* indicated that
VERO or MRC-5 cells do not support the growth of ALVAC-RG
(vCP65); a series of eight (VERO) and 10 (MRC) blind
serial passages caused no detectable adaptation of the
virus to grow in these non avian lines. Analyses of
35 human cell lines (MRC-5, WISH, Detroit 532, HEL, HNK or
EBV-transformed lymphoblastoid cells) infected or
inoculated with ALVAC-RG (vCP65) showed no accumulation
of virus specific DNA suggesting that in these cells the
block in replication occurs prior to DNA synthesis.
Significantly, however, the expression of the rabies
virus glycoprotein gene in all cell lines tested
indicating that the abortive step in the canarypox
replication cycle occurs prior to viral DNA replication.

The safety and efficacy of ALVAC-RG (vCP65) were documented in a series of experiments in animals. A number of species including canaries, chickens, ducks, geese, laboratory rodents (suckling and adult mice), 5 hamsters, guinea-pigs, rabbits, cats and dogs, squirrel monkeys, rhesus macaques and chimpanzees, were inoculated with doses ranging from 10^5 to 10^8 pfu. A variety of routes were used, most commonly subcutaneous, intramuscular and intradermal but also oral (monkeys and 10 mice) and intracerebral (mice).

In canaries, ALVAC-RG (vCP65) caused a "take" lesion at the site of scarification with no indication of disease or death. Intradermal inoculation of rabbits resulted in a typical poxvirus inoculation reaction which 15 did not spread and healed in seven to ten days. There was no adverse side effects due to canarypox in any of the animal tests. Immunogenicity was documented by the development of anti-rabies antibodies following inoculation of ALVAC-RG (vCP65) in rodents, dogs, cats, 20 and primates, as measured by Rapid Fluorescent Focus Inhibition Test (RFFIT). Protection was also demonstrated by rabies virus challenge experiments in mice, dogs, and cats immunized with ALVAC-RG (vCP65).

Volunteers. Twenty-five healthy adults aged 20-45 25 with no previous history of rabies immunization were enrolled. Their health status was assessed by complete medical histories, physical examinations, hematological and blood chemistry analyses. Exclusion criteria included pregnancy, allergies, immune depression of any 30 kind, chronic debilitating disease, cancer, injection of immune globins in the past three months, and seropositivity to human immunodeficiency virus (HIV) or to hepatitis B virus surface antigen.

Study design. Participants were randomly allocated 35 to receive either standard Human Diploid Cell Rabies Vaccine (HDC) batch no E0751 (Pasteur Merieux Serums & Vaccine, Lyon, France) or the study vaccine ALVAC-RG

(vCP65).

The trial was designated as a dose escalation study. Three batches of experimental ALVAC-RG (vCP65) vaccine were used sequentially in three groups of volunteers 5 (Groups A, B and C) with two week intervals between each step. The concentration of the three batches was $10^{3.5}$, $10^{4.5}$, $10^{5.5}$ Tissue Culture Infectious Dose (TCID₅₀) per dose, respectively.

Each volunteer received two doses of the same 10 vaccine subcutaneously in the deltoid region at an interval of four weeks. The nature of the injected vaccine was not known by the participants at the time of the first injection but was known by the investigator.

In order to minimize the risk of immediate 15 hypersensitivity at the time of the second injection, the volunteers of Group B allocated to the medium dose of experimental vaccine were injected 1 h previously with the lower dose and those allocated to the higher dose 20 (Group C) received successively the lower and the medium dose at hourly intervals.

Six months later, the recipients of the highest dosage of ALVAC-RG (vCP65) (Group C) and HDC vaccine were offered a third dose of vaccine; they were then 25 randomized to receive either the same vaccine as previously or the alternate vaccine. As a result, four groups were formed corresponding to the following immunization scheme: 1. HDC, HDC - HDC; 2. HDC, HDC - ALVAC-RG (vCP65); 3. ALVAC-RG (vCP65), ALVAC-RG (vCP65) - HDC; 4. ALVAC-RG (vCP65), ALVAC-RG (vCP65), ALVAC-RG 30 (vCP65).

Monitoring of Side Effects. All subjects were monitored for 1 h after injection and re-examined every day for the next five days. They were asked to record local and systemic reactions for the next three weeks and 35 were questioned by telephone two times a week.

Laboratory Investigators. Blood specimens were obtained before enrollment and two, four and six days

after each injection. Analysis included complete blood cell count, liver enzymes and creatine kinase assays.

5 Antibody assays. Antibody assays were performed seven days prior to the first injection and at days 7, 28, 35, 56, 173, 187 and 208 of the study.

10 The levels of neutralizing antibodies to rabies were determined using the Rapid Fluorescent Focus Inhibition test (RFFIT) (Smith & Yaeger, In Laboratory Techniques on Rabies). Canarypox antibodies were measured by direct 15 ELISA. The antigen, a suspension of purified canarypox virus disrupted with 0.1% Triton X100, was coated in microplates. Fixed dilutions of the sera were reacted for two hours at room temperature and reacting antibodies were revealed with a peroxidase labelled anti-human IgG goat serum. The results are expressed as the optical 20 density read at 490nm.

25 Analysis. Twenty-five subjects were enrolled and completed the study. There were 10 males and 15 females and the mean age was 31.9 (21 to 48). All but three subjects had evidence of previous smallpox vaccination; the three remaining subjects had no typical scar and vaccination history. Three subjects received each of the lower doses of experimental vaccine ($10^{3.5}$ and $10^{4.5}$ TCID₅₀), nine subjects received $10^{5.5}$ TCID₅₀ and ten received the HDC vaccine.

30 Safety (Table 11). During the primary series of immunization, fever greater than 37.7°C was noted within 24 hours after injection in one HDC recipient (37.8°C) and in one vCP65 $10^{5.5}$ TCID₅₀ recipient (38°C). No other 35 systemic reaction attributable to vaccination was observed in any participant.

Local reactions were noted in 9/10 recipients of HDC vaccine injected subcutaneously and in 0/3, 1/3 and 9/9 recipients of vCP65 $10^{3.5}$, $10^{4.5}$, $10^{5.5}$ TCID₅₀, respectively.

Tenderness was the most common symptoms and was always mild. Other local symptoms included redness and

induration which were also mild and transient. All symptoms usually subsided within 24 hours and never lasted more than 72 hours.

There was no significant change in blood cell 5 counts, liver enzymes or creatine kinase values.

Immune Responses; Neutralizing Antibodies to Rabies (Table 12). Twenty eight days after the first injection all the HDC recipients had protective titers (≥ 0.5 IU/ml). By contrast none in groups A and B ($10^{3.5}$ and 10 $10^{4.5}$ TCID₅₀) and only 2/9 in group C ($10^{5.5}$ TCID₅₀) ALVAC-RG (vCP65) recipients reached this protective titer.

At day 56 (i.e. 28 days after the second injection) protective titers were achieved in 0/3 of Group A, 2/3 of Group B and 9/9 of Group C recipients of ALVAC-RG (vCP65) 15 vaccine and persisted in all 10 HDC recipients.

At day 56 the geometric mean titers were 0.05, 0.47, 4.4 and 11.5 IU/ml in groups A, B, C and HDC respectively.

At day 180, the rabies antibody titers had 20 substantially decreased in all subjects but remained above the minimum protective titer of 0.5 IU/ml in 5/10 HCD recipients and in 5/9 ALVAC-RG (vCP65) recipients; the geometric mean titers were 0.51 and 0.45 IU/ml in groups HCD and C, respectively.

Antibodies to the Canarypox virus (Table 13). The 25 pre-immune titers observed varied widely with titers varying from 0.22 to 1.23 O.D. units despite the absence of any previous contact with canary birds in those subjects with the highest titers. When defined as a 30 greater than two-fold increase between preimmunization and post second injection titers, a seroconversion was obtained in 1/3 subjects in group B and in 9/9 subjects in group C whereas no subject seroconverted in groups A or HCD.

Booster Injection. The vaccine was similarly well 35 tolerated six months later, at the time of the booster injection: fever was noted in 2/9 HDC booster recipients

and in 1/10 ALVAC-RG (vCP65) booster recipients. Local reactions were present in 5/9 recipients of HDC booster and in 6/10 recipients of the ALVAC-RG (vCP65) booster.

Observations. FIG. 13 shows graphs of rabies 5 neutralizing antibody titers (Rapid Fluorescent Focus Inhibition Test or RFFIT, IU/ml): Booster effect of HDC and vCP65 ($10^{5.5}$ TCID₅₀) in volunteers previously immunized with either the same or the alternate vaccine. Vaccines were given at days 0, 28 and 180. Antibody 10 titers were measured at days 0, 7, 28, 35, 56, 173, and 187 and 208.

As shown in FIGS. 13A to 13D, the booster dose given resulted in a further increase in rabies antibody titers in every subject whatever the immunization scheme.

15 However, the ALVAC-RG (vCP65) booster globally elicited lower immune responses than the HDC booster and the ALVAC-RG (vCP65), ALVAC-RG (vCP65) - ALVAC-RG (vCP65) group had significantly lower titers than the three other groups. Similarly, the ALVAC-RG (vCP65) booster 20 injection resulted in an increase in canarypox antibody titers in 3/5 subjects who had previously received the HDC vaccine and in all five subjects previously immunized with ALVAC-RG (vCP65).

In general, none of the local side effects from 25 administration of vCP65 was indicative of a local replication of the virus. In particular, lesions of the skin such as those observed after injection of vaccine were absent. In spite of the apparent absence of 30 replication of the virus, the injection resulted in the volunteers generating significant amounts of antibodies to both the canarypox vector and to the expressed rabies glycoprotein.

Rabies neutralizing antibodies were assayed with the 35 Rapid Fluorescent Focus Inhibition Test (RFFIT) which is known to correlate well with the sero neutralization test in mice. Of 9 recipients of $10^{5.5}$ TCID₅₀, five had low level responses after the first dose. Protective titers

of rabies antibodies were obtained after the second injection in all recipients of the highest dose tested and even in 2 of the 3 recipients of the medium dose. In this study, both vaccines were given subcutaneously as 5 usually recommended for live vaccines, but not for the inactivated HDC vaccine. This route of injection was selected as it best allowed a careful examination of the injection site, but this could explain the late appearance of antibodies in HDC recipients: indeed, none 10 of the HDC recipients had an antibody increase at day 7, whereas, in most studies where HDC vaccine is give intramuscularly a significant proportion of subjects do (Klietmann et al., Int'l Green Cross - Geneva, 1981; Kuwert et al., Int'l Green Cross - Geneva, 1981). 15 However, this invention is not necessarily limited to the subcutaneous route of administration..

The GMT (geometric mean titers) of rabies neutralizing antibodies was lower with the investigational vaccine than with the HDC control 20 vaccine, but still well above the minimum titer required for protection. The clear dose effect response obtained with the three dosages used in this study suggest that a higher dosage might induce a stronger response. Certainly from this disclosure the skilled artisan can 25 select an appropriate dosage for a given patient.

The ability to boost the antibody response is another important result of this Example; indeed, an increase in rabies antibody titers was obtained in every subject after the 6 month dose whatever the immunization 30 scheme, showing that preexisting immunity elicited by either the canarypox vector or the rabies glycoprotein had no blocking effect on the booster with the recombinant vaccine candidate or the conventional HDC rabies vaccine. This contrasts findings of others with 35 vaccinia recombinants in humans that immune response may be blocked by pre-existing immunity (Cooney et al., Lancet 1991, 337:567-72; Etlinger et al., Vaccine 9:470-

72, 1991).

Thus, this Example clearly demonstrates that a non-replicating poxvirus can serve as an immunizing vector in humans, with all of the advantages that replicating agents confer on the immune response, but without the safety problem created by a fully permissive virus.

TABLE 11: Reactions in the 5 days following vaccination

vCP65 dosage (TCID50)	10 ^{3.5}		10 ^{4.5}		10 ^{5.5}		H D C control	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
No. vaccinees	3	3	3	3	9	9	10	10
temp >37.7°C	0	0	0	0	0	1	1	0
soreness	0	0	1	1	6	8	8	6
redness	0	0	0	0	0	4	5	4
induration	0	0	0	0	0	4	5	4

TABLE 12: Rabies neutralizing antibodies (REFIT; IU/ml) Individual titers and geometric mean titers (GMT)

	No.	TCID50/dose	Days			
			0	.7	28	35
5	1	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1
	3	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1
	4	$10^{3.5}$	< 0.1	< 0.1	< 0.1	< 0.1
		G.M.T.	< 0.1	< 0.1	< 0.1	< 0.1
	6	$10^{4.5}$	< 0.1	< 0.1	< 0.1	< 0.1
10	7	$10^{4.5}$	< 0.1	< 0.1	< 0.1	2.4
	10	$10^{4.5}$	< 0.1	< 0.1	< 0.1	1.6
		G.M.T.	< 0.1	< 0.1	0.1	0.58
	11	$10^{5.5}$	< 0.1	< 0.1	1.0	3.2
15	13	$10^{5.5}$	< 0.1	< 0.1	0.3	6.0
	14	$10^{5.5}$	< 0.1	< 0.1	0.2	2.1
	17	$10^{5.5}$	< 0.1	< 0.1	< 0.1	1.2
	18	$10^{5.5}$	< 0.1	< 0.1	0.7	8.3
20	20	$10^{5.5}$	< 0.1	< 0.1	< 0.1	0.3
	21	$10^{5.5}$	< 0.1	< 0.1	0.2	2.6
	23	$10^{5.5}$	< 0.1	< 0.1	< 0.1	1.7
	25	$10^{5.5}$	< 0.1	< 0.1	< 0.1	0.6
25		G.M.T.	< 0.1	< 0.1	0.16	1.9
	2	HDC	< 0.1	< 0.1	0.8	7.1
	5	HDC	< 0.1	< 0.1	9.9	12.8
	8	HDC	< 0.1	< 0.1	12.7	21.1
30	9	HDC	< 0.1	< 0.1	6.0	9.9
	12	HDC	< 0.1	< 0.1	5.0	9.2
	15	HDC	< 0.1	< 0.1	2.2	5.2
	16	HDC	< 0.1	< 0.1	2.7	7.7
30	19	HDC	< 0.1	< 0.1	2.6	9.9
	22	HDC	< 0.1	< 0.1	1.4	8.6
	24	HDC	< 0.1	< 0.1	0.8	5.8
		G.M.T.	< 0.1	< 0.1	2.96	9.0
						11.5*

* p = 0.007 student t test

TABLE 13: Canarypox antibodies: ELISA Geometric Mean Titers*

5	vCP65 dosage TCID50/dose	Days				
		0	7	28	35	56
10	$10^{3.5}$	0.69	ND	0.76	ND	0.68
	$10^{4.5}$	0.49	0.45	0.56	0.63	0.87
	$10^{5.5}$	0.38	0.38	0.77	1.42	1.63
	HDC control	0.45	0.39	0.40	0.35	0.39

* optical density at 1/25 dilution

Example 11 - COMPARISON OF THE LD₅₀ OF ALVAC AND NYVAC WITH VARIOUS VACCINIA VIRUS STRAINS

Mice. Male outbred Swiss Webster mice were 5 purchased from Taconic Farms (Germantown, NY) and maintained on mouse chow and water *ad libitum* until use at 3 weeks of age ("normal" mice). Newborn outbred Swiss Webster mice were of both sexes and were obtained following timed pregnancies performed by Taconic Farms. 10 All newborn mice used were delivered within a two day period.

Viruses. ALVAC was derived by plaque purification of a canarypox virus population and was prepared in primary chick embryo fibroblast cells (CEF). Following 15 purification by centrifugation over sucrose density gradients, ALVAC was enumerated for plaque forming units in CEF cells. The WR(L) variant of vaccinia virus was derived by selection of large plaque phenotypes of WR (Panicali et al., 1981). The Wyeth New York State Board 20 of Health vaccine strain of vaccinia virus was obtained from Pharmaceuticals Calf Lymph Type vaccine Dryvax, control number 302001B. Copenhagen strain vaccinia virus VC-2 was obtained from Institut Merieux, France. Vaccinia virus strain NYVAC was derived from Copenhagen 25 VC-2. All vaccinia virus strains except the Wyeth strain were cultivated in Vero African green monkey kidney cells, purified by sucrose gradient density centrifugation and enumerated for plaque forming units on Vero cells. The Wyeth strain was grown in CEF cells and 30 enumerated in CEF cells.

Inoculations. Groups of 10 normal mice were inoculated intracranially (ic) with 0.05 ml of one of several dilutions of virus prepared by 10-fold serially diluting the stock preparations in sterile phosphate-buffered saline. In some instances, undiluted stock 35 virus preparation was used for inoculation.

Groups of 10 newborn mice, 1 to 2 days old, were inoculated ic similarly to the normal mice except that an

injection volume of 0.03 ml was used.

All mice were observed daily for mortality for a period of 14 days (newborn mice) or 21 days (normal mice) after inoculation. Mice found dead the morning following 5 inoculation were excluded due to potential death by trauma.

The lethal dose required to produce mortality for 50% of the experimental population (LD_{50}) was determined by the proportional method of Reed and Muench.

10 Comparison of the LD_{50} of ALVAC and NYVAC with Various Vaccinia Virus Strains for Normal, Young Outbred Mice by the ic Route. In young, normal mice, the virulence of NYVAC and ALVAC were several orders of magnitude lower than the other vaccinia virus strains 15 tested (Table 14). NYVAC and ALVAC were found to be over 3,000 times less virulent in normal mice than the Wyeth strain; over 12,500 times less virulent than the parental VC-2 strain; and over 63,000,000 times less virulent than the WR(L) variant. These results would suggest that 20 NYVAC is highly attenuated compared to other vaccinia strains, and that ALVAC is generally nonvirulent for young mice when administered intracranially, although both may cause mortality in mice at extremely high doses (3.85x10⁸ PFUs, ALVAC and 3x10⁸ PFUs, NYVAC) by an 25 undetermined mechanism by this route of inoculation.

25 Comparison of the LD_{50} of ALVAC and NYVAC with Various Vaccinia Virus Strains for Newborn Outbred Mice by the ic Route. The relative virulence of 5 poxvirus strains for normal, newborn mice was tested by titration 30 in an intracranial (ic) challenge model system (Table 15). With mortality as the endpoint, LD_{50} values indicated that ALVAC is over 100,000 times less virulent 35 than the Wyeth vaccine strain of vaccinia virus; over 200,000 times less virulent than the Copenhagen VC-2 strain of vaccinia virus; and over 25,000,000 times less virulent than the WR-L variant of vaccinia virus. Nonetheless, at the highest dose tested, 6.3x10⁷ PFUs,

100% mortality resulted. Mortality rates of 33.3% were observed at 6.3×10^6 PFUs. The cause of death, while not actually determined, was not likely of toxicological or traumatic nature since the mean survival time (MST) of 5 mice of the highest dosage group (approximately 6.3 LD_{50}) was 6.7 ± 1.5 days. When compared to WR(L) at a challenge dose of 5 LD_{50} , wherein MST is 4.8 ± 0.6 days, the MST of ALVAC challenged mice was significantly longer ($P=0.001$).

10 Relative to NYVAC, Wyeth was found to be over 15,000 times more virulent; VC-2, greater than 35,000 times more virulent; and WR(L), over 3,000,000 times more virulent. Similar to ALVAC, the two highest doses of NYVAC, 6×10^8 and 6×10^7 PFUs, caused 100% mortality. However, the MST 15 of mice challenged with the highest dose, corresponding to 380 LD_{50} , was only 2 days (9 deaths on day 2 and 1 on day 4). In contrast, all mice challenged with the highest dose of WR-L, equivalent to 500 LD_{50} , survived to day 4.

Table 14. Calculated 50% Lethal Dose for mice by various vaccinia virus strains and for canarypox virus (ALVAC) by the ic route.

	POXVIRUS STRAIN	CALCULATED LD ₅₀ (PFUs)
5	WR(L)	2.5
10	VC-2	1.26x10 ⁴
15	WYETH	5.00x10 ⁴
	NYVAC	1.58x10 ⁸
20	ALVAC	1.58x10 ⁸

Table 15. Calculated 50% Lethal Dose for newborn mice by various vaccinia virus strains and for canarypox virus (ALVAC) by the ic route.

	POXVIRUS STRAIN	CALCULATED LD ₅₀ (PFUs)
25	WR(L)	0.4
30	VC-2	0.1
	WYETH	1.6
35	NYVAC	1.58x10 ⁶
	ALVAC	1.00x10 ⁷

Example 12 - EVALUATION OF NYVAC (vP866) AND NYVAC-RG (vP879)

Immunoprecipitations. Preformed monolayers of avian 5 or non-avian cells were inoculated with 10 pfu per cell of parental NYVAC (vP866) or NYVAC-RG (vP879) virus. The inoculation was performed in EMEM free of methionine and supplemented with 2% dialyzed fetal bovine serum. After a one hour incubation, the inoculum was removed and the 10 medium replaced with EMEM (methionine free) containing 20 μ Ci/ml of 35 S-methionine. After an overnight incubation of approximately 16 hours, cells were lysed by the 15 addition of Buffer A (1% Nonidet P-40, 10 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 0.01% sodium azide, 500 units per ml of aprotinin, and 0.02% phenyl methyl sulfonyl fluoride). Immunoprecipitation was performed using a rabies glycoprotein specific monoclonal antibody designated 24-3F10 supplied by Dr. C. Trinarchi, Griffith Laboratories, New York State Department of Health, 20 Albany, New York, and a rat anti-mouse conjugate obtained from Boehringer Mannheim Corporation (Cat. #605-500). Protein A Sepharose CL-48 obtained from Pharmacia LKB Biotechnology Inc., Piscataway, New Jersey, was used as a support matrix. Immunoprecipitates were fractionated on 25 10% polyacrylamide gels according to the method of Dreyfuss et. al. (1984). Gels were fixed, treated for fluorography with 1M Na-salicylate for one hour, and exposed to Kodak XAR-2 film to visualize the immunoprecipitated protein species.

30 Sources of Animals. New Zealand White rabbits were obtained from Hare-Marland (Hewitt, New Jersey). Three week old male Swiss Webster outbred mice, timed pregnant female Swiss Webster outbred mice, and four week old Swiss Webster nude (nu^+nu^+) mice were obtained from 35 Taconic Farms, Inc. (Germantown, New York). All animals were maintained according to NIH guidelines. All animal protocols were approved by the institutional IACUC. When deemed necessary, mice which were obviously terminally

ill were euthanized.

Evaluation of Lesions in Rabbits. Each of two rabbits was inoculated intradermally at multiple sites with 0.1 ml of PBS containing 10^4 , 10^5 , 10^6 , 10^7 , or 10^8 pfu of each test virus or with PBS alone. The rabbits 5 were observed daily from day 4 until lesion resolution. Indurations and ulcerations were measured and recorded.

Virus Recovery from Inoculation Sites. A single rabbit was inoculated intradermally at multiple sites of 10 0.1 ml of PBS containing 10^6 , 10^7 , or 10^8 pfu of each test virus or with PBS alone. After 11 days, the rabbit was euthanized and skin biopsy specimens taken from each of 15 the inoculation sites were aseptically prepared by mechanical disruption and indirect sonication for virus recovery. Infectious virus was assayed by plaque titration on CEF monolayers.

Virulence in Mice. Groups of ten mice, or five in the nude mice experiment, were inoculated ip with one of several dilutions of virus in 0.5 ml of sterile PBS. 20 Reference is also made to Example 11.

Cyclophosphamide (CY) Treatment. Mice were injected by the ip route with 4 mg (0.02 ml) of CY (SIGMA) on day -2, followed by virus injection on day 0. On the following days post infection, mice were injected ip with 25 CY: 4 mg on day 1; 2 mg on days 4, 7 and 11; 3 mg on days 14, 18, 21, 25 and 28. Immunosuppression was indirectly monitored by enumerating white blood cells with a Coulter Counter on day 11. The average white blood cell count was 13,500 cells per μ l for untreated mice (n=4) and 30 4,220 cells per μ l for CY-treated control mice (n=5).

Calculation of LD₅₀. The lethal dose required to produce 50% mortality (LD₅₀) was determined by the proportional method of Reed and Muench (Reed and Muench 1938).

35 Potency Testing of NYVAC-RG in Mice. Four to six week old mice were inoculated in the footpad with 50 to 100 μ l of a range of dilutions (2.0 - 8.0 log₁₀ tissue

culture infective dose 50% (TCID₅₀) of either VV-RG (Kieny et al., 1984), ALVAC-RG (Taylor et al., 1991b), or the NYVAC-RG. Each group consisted of eight mice. At 14 days post-vaccination, the mice were challenged by 5 intracranial inoculation with 15 LD₅₀ of the rabies virus CVS strain (0.03 ml). On day 28, surviving mice were counted and protective does 50% (PD₅₀) calculated.

Derivation of NYVAC (vP866). The NYVAC strain of vaccinia virus was generated from VC-2, a plaque cloned 10 isolate of the COPENHAGEN vaccine strain. To generate NYVAC from VC-2, eighteen vaccinia ORFs, including a number of viral functions associated with virulence, were precisely deleted in a series of sequential manipulations as described earlier in this disclosure. These deletions 15 were constructed in a manner designed to prevent the appearance of novel unwanted open reading frames. FIG. 10 schematically depicts the ORFs deleted to generate NYVAC. At the top of FIG. 10 is depicted the HindIII restriction map of the vaccinia virus genome (VC-2 plaque 20 isolate, COPENHAGEN strain). Expanded are the six regions of VC-2 that were sequentially deleted in the generation of NYVAC. The deletions were described earlier in this disclosure (Examples 1 through 6). Below such deletion locus is listed the ORFs which were deleted 25 from that locus, along with the functions or homologies and molecular weight of their gene products.

Replication Studies of NYVAC and ALVAC on Human Tissue Cell Lines. In order to determine the level of replication of NYVAC strain of vaccinia virus (vP866) in 30 cells of human origin, six cell lines were inoculated at an input multiplicity of 0.1 pfu per cell under liquid culture and incubated for 72 hours. The COPENHAGEN parental clone (VC-2) was inoculated in parallel. Primary chick embryo fibroblast (CEF) cells (obtained 35 from 10-11 day old embryonated eggs of SPF origin, Spafas, Inc., Storrs, CT) were included to represent a permissive cell substrate for all viruses. Cultures were

analyzed on the basis of two criteria: the occurrence of productive viral replication and expression of an extrinsic antigen.

The replication potential of NYVAC in a number of 5 human derived cells are shown in Table 16. Both VC-2 and NYVAC are capable of productive replication in CEF cells, although NYVAC with slightly reduced yields. VC-2 is also capable of productive replication in the six human derived cell lines tested with comparable yields except 10 in the EBV transformed lymphoblastoid cell line JT-1 (human lymphoblastoid cell line transformed with Epstein-Barr virus, see Rickinson et al., 1984). In contrast, NYVAC is highly attenuated in its ability to productively replicate in any of the human derived cell lines tested. 15 Small increases of infectious virus above residual virus levels were obtained from NYVAC-infected MRC-5 (ATCC #CCL171, human embryonic lung origin), DETROIT 532 (ATCC #CCL54, human foreskin, Downs Syndrome), HEL 299 (ATCC #CCL137, human embryonic lung cells) and HNK (human 20 neonatal kidney cells, Whittaker Bioproducts, Inc. Walkersville, MD, Cat #70-151) cells. Replication on these cell lines was significantly reduced when compared to virus yields obtained from NYVAC-infected CEF cells or with parental VC-2 (Table 16). It should be noted that 25 the yields at 24 hours in CEF cells for both NYVAC and VC-2 is equivalent to the 72-hour yield. Allowing the human cell line cultures to incubate an additional 48 hours (another two viral growth cycles) may, therefore, have amplified the relative virus yield obtained. 30 Consistent with the low levels of virus yields obtained in the human-derived cell lines, MRC-5 and DETROIT 532, detectable but reduced levels of NYVAC-specific DNA accumulation were noted. The level of DNA accumulation in the MRC-5 and DETROIT 532 NYVAC-infected 35 cell lines relative to that observed in NYVAC-infected CEF cells paralleled the relative virus yields. NYVAC-specific viral DNA accumulation was not observed in any

of the other human-derived cells.

An equivalent experiment was also performed using the avipox virus, ALVAC. The results of virus replication are also shown in Table 16. No progeny virus was detectable in any of the human cell lines consistent with the host range restriction of canarypox virus to avian species. Also consistent with a lack of productive replication of ALVAC in these human-derived cells is the observation that no ALVAC-specific DNA accumulation was detectable in any of the human-derived cell lines.

Expression of Rabies Glycoprotein by NYVAC-RG (vP879) in Human Cells. In order to determine whether efficient expression of a foreign gene could be obtained in the absence of significant levels of productive viral replication, the same cell lines were inoculated with the NYVAC recombinant expressing the rabies virus glycoprotein (vP879, Example 7) in the presence of ³⁵S-methionine. Immunoprecipitation of the rabies glycoprotein was performed from the radiolabelled culture lysate using a monoclonal antibody specific for the rabies glycoprotein. Immunoprecipitation of a 67kDa protein was detected consistent with a fully glycosylated form of the rabies glycoprotein. No serologically crossreactive product was detected in uninfected or parental NYVAC infected cell lysates. Equivalent results were obtained with all other human cells analyzed.

Inoculations on the Rabbit Skin. The induction and nature of skin lesions on rabbits following intradermal (id) inoculations has been previously used as a measure of pathogenicity of vaccinia virus strains (Buller et al., 1988; Child et al., 1990; Fenner, 1958, Flexner et al., 1987; Ghendon and Chernos 1964). Therefore, the nature of lesions associated with id inoculations with the vaccinia strains WR (ATCC #VR119 plaque purified on 30 CV-1 cells, ATCC #CCL70, and a plaque isolate designated L variant, ATCC #VR2035 selected, as described in Panicali et al., 1981)), WYETH (ATCC #VR325 marketed as

DRYVAC by Wyeth Laboratories, Marietta, PA), COPENHAGEN (VC-2), and NYVAC was evaluated by inoculation of two rabbits (A069 and A128). The two rabbits displayed different overall sensitivities to the viruses, with 5 rabbit A128 displaying less severe reactions than rabbit A069. In rabbit A128, lesions were relatively small and resolved by 27 days post-inoculation. On rabbit A069, lesions were intense, especially for the WR inoculation sites, and resolved only after 49 days. Intensity of the 10 lesions was also dependent on the location of the inoculation sites relative to the lymph drainage network. In particular, all sites located above the backspine displayed more intense lesions and required longer times to resolve the lesions located on the flanks. All 15 lesions were measured daily from day 4 to the disappearance of the last lesion, and the means of maximum lesion size and days to resolution were calculated (Table 17). No local reactions were observed from sites injected with the control PBS. Ulcerative 20 lesions were observed at sites injected with WR, VC-2 and WYETH vaccinia virus strains. Significantly, no induration or ulcerative lesions were observed at sites of inoculation with NYVAC.

Persistence of Infectious Virus at the Site of
25 Inoculation. To assess the relative persistence of these viruses at the site of inoculation, a rabbit was inoculated intradermally at multiple sites with 0.1 ml PBS containing 10^6 , 10^7 or 10^8 pfu of VC-2, WR, WYETH or NYVAC. For each virus, the 10^7 pfu dose was located above 30 the backspine, flanked by the 10^6 and 10^8 doses. Sites of inoculation were observed daily for 11 days. WR elicited the most intense response, followed by VC-2 and WYETH (Table 18). Ulceration was first observed at day 9 for WR and WYETH and day 10 for VC-2. Sites inoculated with 35 NYVAC or control PBS displayed no induration or ulceration. At day 11 after inoculation, skin samples from the sites of inoculation were excised, mechanically

disrupted, and virus was titrated on CEF cells. The results are shown in Table 18. In no case was more virus recovered at this timepoint than was administered.

Recovery of vaccinia strain, WR, was approximately 10^6 pfu of virus at each site irrespective of amount of virus administered. Recovery of vaccinia strains WYETH and VC-2 was 10^3 to 10^4 pfu regardless of amount administered. No infectious virus was recovered from sites inoculated with NYVAC.

10 Inoculation of Genetically or Chemically Immune Deficient Mice. Intraperitoneal inoculation of high doses of NYVAC (5×10^8 pfu) or ALVAC (10^9 pfu) into nude mice caused no deaths, no lesions, and no apparent disease through the 100 day observation period. In 15 contrast, mice inoculated with WR (10^3 to 10^4 pfu), WYETH (5×10^7 or 5×10^8 pfu) or VC-2 (10^4 to 10^9 pfu) displayed disseminated lesions typical of poxviruses first on the toes, then on the tail, followed by severe orchitis in some animals. In mice infected with WR or 20 WYETH, the appearance of disseminated lesions generally led to eventual death, whereas most mice infected with VC-2 eventually recovered. Calculated LD₅₀ values are given in Table 19.

25 In particular, mice inoculated with VC-2 began to display lesions on their toes (red papules) and 1 to 2 days later on the tail. These lesions occurred between 11 and 13 days post-inoculation (pi) in mice given the highest doses (10^9 , 10^8 , 10^7 and 10^6 pfu), on day 16 pi in mice given 10^5 pfu and on day 21 pi in mice given 10^4 pfu. 30 No lesions were observed in mice inoculated with 10^3 and 10^2 pfu during the 100 day observation period. Orchitis was noticed on day 23 pi in mice given 10^9 and 10^8 pfu, and approximately 7 days later in the other groups (10^7 to 10^4 pfu). Orchitis was especially intense in the 10^9 and 35 10^8 pfu groups and, although receding, was observed until the end of the 100 day observation period. Some pox-like lesions were noticed on the skin of a few mice, occurring

around 30-35 days pi. Most pox lesions healed normally between 60-90 days pi. Only one mouse died in the group inoculated with 10^9 pfu (Day 34 pi) and one mouse died in the group inoculated with 10^8 pfu (Day 94 pi). No other 5 deaths were observed in the VC-2 inoculated mice.

Mice inoculated with 10^4 pfu of the WR strain of vaccinia started to display pox lesions on Day 17 pi. These lesions appeared identical to the lesions displayed by the VC-2 injected mice (swollen toes, tail). Mice 10 inoculated with 10^3 pfu of the WR strain did not develop lesions until 34 days pi. Orchitis was noticed only in the mice inoculated with the highest dose of WR (10^4 pfu). During the latter stages of the observation period, lesions appeared around the mouth and the mice 15 stopped eating. All mice inoculated with 10^4 pfu of WR died or were euthanized when deemed necessary between 21 days and 31 days pi. Four out of the 5 mice injected with 10^3 pfu of WR died or were euthanized when deemed necessary between 35 days and 57 days pi. No deaths were 20 observed in mice inoculated with lower doses of WR (1 to 100 pfu).

Mice inoculated with the WYETH strain of vaccinia virus at higher doses (5×10^7 and 5×10^8 pfu) showed lesions on toes and tails, developed orchitis, and died. 25 Mice injected with 5×10^6 pfu or less of WYETH showed no signs of disease or lesions.

As shown in Table 19, CY-treated mice provided a more sensitive model for assaying poxvirus virulence than did nude mice. LD₅₀ values for the WR, WYETH, and VC-2 30 vaccinia virus strains were significantly lower in this model system than in the nude mouse model. Additionally, lesions developed in mice injected with WYETH, WR and VC-2 vaccinia viruses, as noted below, with higher doses of each virus resulting in more rapid formation of lesions. 35 As was seen with nude mice, CY-treated mice injected with NYVAC or ALVAC did not develop lesions. However, unlike nude mice, some deaths were observed in CY-treated mice

challenged with NYVAC or ALVAC, regardless of the dose. These random incidences are suspect as to the cause of death.

Mice injected with all doses of WYETH (9.5×10^4 to 5 9.5×10^8 pfu) displayed pox lesions on their tail and/or on their toes between 7 and 15 days pi. In addition, the tails and toes were swollen. Evolution of lesions on the tail was typical of pox lesions with formation of a papule, ulceration and finally formation of a scab. Mice 10 inoculated with all doses of VC-2 (1.65×10^5 to 1.65×10^9) also developed pox lesions on their tails and/or their toes analogous to those of WYETH injected mice. These lesions were observed between 7-12 days post 15 inoculation. No lesions were observed on mice injected with lower doses of WR virus, although deaths occurred in these groups.

Potency Testing of NYVAC-RG. In order to determine that attenuation of the COPENHAGEN strain of vaccinia virus had been effected without significantly altering 20 the ability of the resulting NYVAC strain to be a useful vector, comparative potency tests were performed. In order to monitor the immunogenic potential of the vector during the sequential genetic manipulations performed to attenuate the virus, a rabiesvirus glycoprotein was used 25 as a reporter extrinsic antigen. The protective efficacy of the vectors expressing the rabies glycoprotein gene was evaluated in the standard NIH mouse potency test for rabies (Seligmann, 1973). Table 20 demonstrates that the PD₅₀ values obtained with the highly attenuated NYVAC 30 vector are identical to those obtained using a COPENHAGEN-based recombinant containing the rabies glycoprotein gene in the tk locus (Kieny et al., 1984) and similar to PD₅₀ values obtained with ALVAC-RG, a canarypox based vector restricted to replication to avian 35 species.

Observations. NYVAC, deleted of known virulence genes and having restricted *in vitro* growth

characteristics, was analyzed in animal model systems to assess its attenuation characteristics. These studies were performed in comparison with the neurovirulent vaccinia virus laboratory strain, WR, two vaccinia virus 5 vaccine strains, WYETH (New York City Board of Health) and COPENHAGEN (VC-2), as well as with a canarypox virus strain, ALVAC (See also Example 11). Together, these viruses provided a spectrum of relative pathogenic potentials in the mouse challenge model and the rabbit 10 skin model, with WR being the most virulent strain, WYETH and COPENHAGEN (VC-2) providing previously utilized attenuated vaccine strains with documented characteristics, and ALVAC providing an example of a poxvirus whose replication is restricted to avian 15 species. Results from these *in vivo* analyses clearly demonstrate the highly attenuated properties of NYVAC relative to the vaccinia virus strains, WR, WYETH and COPENHAGEN (VC-2) (Tables 14-20). Significantly, the LD₅₀ values for NYVAC were comparable to those observed with 20 the avian host restricted avipoxvirus, ALVAC. Deaths due to NYVAC, as well as ALVAC, were observed only when extremely high doses of virus were administered via the intracranial route (Example 11, Tables 14, 15, 19). It has not yet been established whether these deaths were 25 due to nonspecific consequences of inoculation of a high protein mass. Results from analyses in immunocompromised mouse models (nude and CY-treated) also demonstrate the relatively high attenuation characteristics of NYVAC, as compared to WR, WYETH and COPENHAGEN strains (Tables 17 30 and 18). Significantly, no evidence of disseminated vaccinia infection or vaccinia disease was observed in NYVAC-inoculated animals or ALVAC-inoculated animals over the observation period. The deletion of multiple virulence-associated genes in NYVAC shows a synergistic 35 effect with respect to pathogenicity. Another measure of the innocuity of NYVAC was provided by the intradermal administration on rabbit skin (Tables 17 and 18).

Considering the results with ALVAC, a virus unable to replicate in nonavian species, the ability to replicate at the site of inoculation is not the sole correlate with reactivity, since intradermal inoculation of ALVAC caused 5 areas of induration in a dose dependent manner.

Therefore, it is likely that factors other than the replicative capacity of the virus contribute to the formation of the lesions. Deletion of genes in NYVAC prevents lesion occurrence.

10 Together, the results in this Example and in foregoing Examples, including Example 11, demonstrate the highly attenuated nature of NYVAC relative to WR, and the previously utilized vaccinia virus vaccine strains, WYETH and COPENHAGEN. In fact, the pathogenic profile of 15 NYVAC, in the animal model systems tested, was similar to that of ALVAC, a poxvirus known to productively replicate only in avian species. The apparently restricted capacity of NYVAC to productively replicate on cells derived from humans (Table 16) and other species, 20 including the mouse, swine, dog and horse, provides a considerable barrier that limits or prevents potential transmission to unvaccinated contacts or to the general environment in addition to providing a vector with reduced probability of dissemination within the 25 vaccinated individual.

Significantly, NYVAC-based vaccine candidates have been shown to be efficacious. NYVAC recombinants expressing foreign gene products from a number of pathogens have elicited immunological responses towards 30 the foreign gene products in several animal species, including primates. In particular, a NYVAC-based recombinant expressing the rabies glycoprotein was able to protect mice against a lethal rabies challenge. The potency of the NYVAC-based rabies glycoprotein 35 recombinant was comparable to the PD₅₀ value for a COPENHAGEN-based recombinant containing the rabies glycoprotein in the tk locus (Table 20). NYVAC-based

recombinants have also been shown to elicit measles virus neutralizing antibodies in rabbits and protection against pseudorabies virus and Japanese encephalitis virus challenge in swine. The highly attenuated NYVAC strain 5 confers safety advantages with human and veterinary applications (Tartaglia et al., 1990). Furthermore, the use of NYVAC as a general laboratory expression vector system may greatly reduce the biological hazards associated with using vaccinia virus.

10 By the following criteria, the results of this Example and the Examples herein, including Example 11, show NYVAC to be highly attenuated: a) no detectable induration or ulceration at site of inoculation (rabbit skin); b) rapid clearance of infectious virus from 15 intradermal site of inoculation (rabbit skin); c) absence of testicular inflammation (nude mice); d) greatly reduced virulence (intracranial challenge, both three-week old and newborn mice); e) greatly reduced pathogenicity and failure to disseminate in 20 immunodeficient subjects (nude and cyclophosphamide treated mice); and f) dramatically reduced ability to replicate on a variety of human tissue culture cells. Yet, in spite of being highly attenuated, NYVAC, as a 25 vector, retains the ability to induce strong immune responses to extrinsic antigens.

TABLE 16 Replication of COPENHAGEN (VC-2), NYVAC and ALVAC in avian or human derived cell lines

Cells	Hours post-infection	Yield ^a			% Yield
		VC-2	NYVAC	ALVAC	
5	0	3.8 ^b	3.7	4.5	
	24	8.3	7.8	6.6	
	48	8.6	7.9	7.7	
	72	8.3	7.7	7.5	25
	72A ^c	<1.4	1.8	3.1	
10	MRC-5	0	3.8	3.8	4.7
		72	7.2	4.6	3.8
		72A	2.2	2.2	3.7
	WISH*	0	3.4	3.4	4.3
		72	7.6	2.2	3.1
		72A	— ^d	1.9	2.9
15	DETROIT	0	3.8	3.7	4.4
		72	7.2	5.4	3.4
		72A	1.7	1.7	2.9
	HEL	0	3.8	3.5	4.3
		72	7.5	4.6	3.3
		72A	2.5	2.1	3.6
20	JT-1	0	3.1	3.1	4.1
		72	6.5	3.1	4.2
		72A	2.4	2.1	4.4
	HNK	0	3.8	3.7	4.7
		72	7.6	4.5	3.6
		72A	3.1	2.7	3.7
25					

a: Yield of NYVAC at 72 hours post-infection expressed as a percentage of yield of VAC-2 after 72 hours on the same cell line.

b: Titer expressed as LOG_{50} pfu per ml.

c: Sample was incubated in the presence of 40 $\mu\text{g}/\text{ml}$ of cytosine arabinoside.

d: Not determined.

*: ATCC #CCL25 Human amniotic cells.

Table 17. Induration and ulceration at the site of intradermal inoculation of the rabbit skin

5	VIRUS STRAIN	DOSE ^a	INDURATION		ULCERATION		
			Size ^b	Days ^c	Size	Days	
10	WR	10^4	386	30	88	30	
		10^5	622	35	149	32	
		10^6	1057	34	271	34	
	WYETH	10^7	877	35	204	35	
		10^8	581	25	88	26	
	VC-2	10^4	32	5	-- ^d	--	
15		10^5	116	15	--	--	
		10^6	267	17	3	15	
		10^7	202	17	3	24	
NYVAC	10^8	240	29	12	31		
	10^4	64	7	--	--		
	10^5	86	8	--	--		
20	VC-2	10^6	136	17	--	--	
		10^7	167	21	6	10	
		10^8	155	32	6	8	
	NYVAC	10^4	--	--	--	--	
		10^5	--	--	--	--	
		10^6	--	--	--	--	
25	NYVAC	10^7	--	--	--	--	
		10^8	--	--	--	--	

^a pfu of indicated vaccinia virus in 0.1 ml PBS inoculated intradermally into one site.

^b mean maximum size of lesions (mm²)

^c mean time after inoculation for complete healing of lesion.

^d no lesions discernable.

Table 18. Persistence of poxviruses at the site of intradermal inoculation

	Virus	Inoculum Dose	Total Virus Recovered
5	WR	8.0 ^a	6.14
		7.0	6.26
		6.0	6.21
10	WYETH	8.0	3.66
		7.0	4.10
		6.0	3.59
15	VC-2	8.0	4.47
		7.0	4.74
		6.0	3.97
20	NYVAC	8.0	0
		7.0	0
		6.0	0

a: expressed as \log_{10} pfu.

Table 19. Virulence studies in immunocompromised mice

Poxvirus Strain	LD ₅₀ ^a	
	Nude mice	Cyclophosphamide treated mice
WR	422	42
VC-2	>10 ⁹	<1.65 x 10 ⁵
WYETH	1.58 x 10 ⁷	1.83 x 10 ⁶
NYVAC	>5.50 x 10 ⁸	7.23 x 10 ⁸
ALVAC	>10 ⁹	≥5.00 x 10 ^{8b}

10

a: Calculated 50% lethal dose (pfu) for nude or cyclophosphamide treated mice by the indicated vaccinia viruses and for ALVAC by intraperitoneal route.

15

b: 5 out of 10 mice died at the highest dose of 5 x 10⁸ pfu.

20

Table 20. Comparative efficacy of NYVAC-RG and ALVAC-RG in mice

25

Recombinant	PD ₅₀ ^a
VV-RG	3.74
ALVAC-RG	3.86
NYVAC-RG	3.70

30

a: Four to six week old mice were inoculated in the footpad with 50-100 μ l of a range of dilutions (2.0 - 8.0 log₁₀ tissue culture infection dose 50% (TCID₅₀) of either the VV-RG (Kieny et al., 1984), ALVAC-RG (vCP65) or NYVAC-RG (vP879). At day 14, mice of each group were challenged by intracranial inoculation of 30 μ l of a live CVS strain rabies virus corresponding to 15 lethal dose 50% (LD₅₀) per mouse. At day 28, surviving mice were counted and a protective dose 50% (PD₅₀) was calculated.

Example 13 - CONSTRUCTION OF TROVAC RECOMBINANTS
EXPRESSING THE HEMAGGLUTININ GLYCOPROTEINS
OF AVIAN INFLUENZA VIRUSES

5 This Example describes the development of fowlpox virus recombinants expressing the hemagglutinin genes of three serotypes of avian influenza virus.

Cells and Viruses. Plasmids containing cDNA clones of the H4, H5 and H7 hemagglutinin genes were obtained 10 from Dr. Robert Webster, St. Jude Children's Research Hospital, Memphis, Tennessee. The strain of FPV designated FP-1 has been described previously (Taylor et al., 1988a, b). It is a vaccine strain useful in vaccination of day old chickens. The parental virus 15 strain Duvette was obtained in France as a fowlpox scab from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chick embryo fibroblast (CEF) cells. This virus was obtained in September 1980 20 by Rhone Merieux, Lyon, France, and a master viral seed established. The virus was received by Virogenetics in September 1989, where it was subjected to four successive plaque purifications. One plaque isolate was further amplified in primary CEF cells and a stock virus, 25 designated as TROVAC, was established. The stock virus used in the in vitro recombination test to produce TROVAC-AIH5 (vFP89) and TROVAC-AIH4 (vFP92) had been further amplified though 8 passages in primary CEF cells. The stock virus used to produce TROVAC-AIH7 (vFP100) had 30 been further amplified through 12 passages in primary CEF cells.

Construction of Fowlpox Insertion Plasmid at F8 Locus. Plasmid pRW731.15 contains a 10 kbp PvuII-PvuII fragment cloned from TROVAC genomic DNA. The nucleotide 35 sequence was determined on both strands for a 3659 bp PvuII-EcoRV fragment. This sequence is shown in FIG. 11 (SEQ ID NO:77). The limits of an open reading frame designated in this laboratory as F8 were determined within this sequence. The open reading frame is

initiated at position 495 and terminates at position 1887. A deletion was made from position 779 to position 1926, as described below.

5 Plasmid pRW761 is a sub-clone of pRW731.15 containing a 2430 bp EcoRV-EcoRV fragment. Plasmid pRW761 was completely digested with XbaI and partially digested with SspI. A 3700 bp XbaI-SspI band was isolated and ligated with the annealed double-stranded oligonucleotides JCA017 (SEQ ID NO:37) and JCA018 (SEQ ID 10 NO:38).

JCA017 (SEQ ID NO:37) 5' CTAGACACTTATGTTTTAATATCCGGTCTT

AAAAGCTTCCCGGGATCCTTATACGGGAATAAT 3'

JCA018 (SEQ ID NO:38) 5' ATTATTCCCCGTATAAGGATCCCCCGGGAA

GCTTTTAAGACCGGATATTAAAAAACATAAAAGTGT 3'

15 The plasmid resulting from this ligation was designated pJCA002. Plasmid pJCA004 contains a non-pertinent gene linked to the vaccinia virus H6 promoter in plasmid pJCA002. The sequence of the vaccinia virus H6 promoter has been previously described (Taylor et al., 20 1988a, b; Guo et al. 1989; Perkus et al., 1989). Plasmid pJCA004 was digested with EcoRV and BamHI which deletes the non-pertinent gene and a portion of the 3' end of the H6 promoter. Annealed oligonucleotides RW178 (SEQ ID NO:48) and RW179 (SEQ ID NO:49) were cut with EcoRV and 25 BamHI and inserted between the EcoRV and BamHI sites of JCA004 to form pRW846.

RW178 (SEQ ID NO:48): 5' TCATTATCGCGATATCCGTGTTAACTAGCTA
GCTAATTTTATCCCCGGATCCTTATCA 3'

RW179 (SEQ ID NO:49): 5' GTATAAGGATCCCGGGATAAAAATTAGCT
AGCTAGTTAACACGGATATCGCGATAATGA 3'

30 Plasmid pRW846 therefore contains the H6 promoter 5' of EcoRV in the de-ORFed F8 locus. The HincII site 3' of the H6 promoter in pRW846 is followed by translation stop codons, a transcriptional stop sequence recognized by 35 vaccinia virus early promoters (Yuen et al., 1987) and a SmaI site.

Construction of Fowlpox Insertion Plasmid at F7 Locus. The original F7 non-de-ORFed insertion plasmid, pRW731.13, contained a 5.5 kb FP genomic PvuII fragment in the PvuII site of pUC9. The insertion site was a 5 unique HincII site within these sequences. The nucleotide sequence shown in FIG. 12 (SEQ ID NO:78) was determined for a 2356 bp region encompassing the unique HincII site. Analysis of this sequence revealed that the unique HincII site (FIG. 12, underlined) was situated 10 within an ORF encoding a polypeptide of 90 amino acids. The ORF begins with an ATG at position 1531 and terminates at position 898 (positions marked by arrows in FIG. 12).

The arms for the de-ORFed insertion plasmid were 15 derived by PCR using pRW731.13 as template. A 596 bp arm (designated as HB) corresponding to the region upstream from the ORF was amplified with oligonucleotides F73PH2 (SEQ ID NO:50) (5'-GACAATCTAACGTCTATATTAGAC-3') and F73PB (SEQ ID NO:51) (5'-GGATTTTAGGTAGACAC-3'). A 20 270 bp arm (designated as EH) corresponding to the region downstream from the ORF was amplified using oligonucleotides F75PE (SEQ ID NO:52) (5'-TCATCGTCTTCATCATCG-3') and F73PH1 (SEQ ID NO:53) (5'-GTCTTAAACTTATTGTAAGGGTATAACCTG-3').

25 Fragment EH was digested with EcoRV to generate a 126 bp fragment. The EcoRV site is at the 3'-end and the 5'-end was formed, by PCR, to contain the 3' end of a HincII site. This fragment was inserted into pBS-SK (Stratagene, La Jolla, CA) digested with HincII to form 30 plasmid pF7D1. The sequence was confirmed by dideoxynucleotide sequence analysis. The plasmid pF7D1 was linearized with ApaI, blunt-ended using T4 DNA polymerase, and ligated to the 596 bp HB fragment. The resultant plasmid was designated as pF7D2. The entire 35 sequence and orientation were confirmed by nucleotide sequence analysis.

The plasmid pF7D2 was digested with EcoRV and BglII

to generate a 600 bp fragment. This fragment was inserted into pBS-SK that was digested with ApaI, blunt-ended with T4 DNA polymerase, and subsequently digested with BamHI. The resultant plasmid was designated as 5 pF7D3. This plasmid contains an HB arm of 404 bp and a EH arm of 126 bp.

The plasmid pF7D3 was linearized with XhoI and blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. This linearized 10 plasmid was ligated with annealed oligonucleotides F7MCSB (SEQ ID NO:54) (5'- AACGATTAGTTAGTTACTAAAGCTGCTGCAGCCGGGTTTTATTAGTTAGTT 15 AGTC-3') and F7MCSA (SEQ ID NO:55) (5'- GACTAACTAACTAATAAAAAAA 20 15 CCCGGGCTGCAGCAAGCTTTGTAACTAACTAATCGTT-3'). This was performed to insert a multiple cloning region containing the restriction sites for HindIII, PstI and SmaI between the EH and HB arms. The resultant plasmid was designated as pF7D0.

20 Construction of Insertion Plasmid for the H4 Hemagglutinin at the F8 Locus. A cDNA copy encoding the avian influenza H4 derived from A/Ty/Min/833/80 was obtained from Dr. R. Webster in plasmid pTM4H833. The plasmid was digested with HindIII and NruI and blunt-ended using the Klenow fragment of DNA polymerase in the 25 presence of dNTPs. The blunt-ended 2.5 kbp HindIII-NruI fragment containing the H4 coding region was inserted into the HincII site of pIBI25 (International Biotechnologies, Inc., New Haven, CT). The resulting 30 plasmid pRW828 was partially cut with BanII, the linear product isolated and recut with HindIII. Plasmid pRW828 now with a 100 bp HindIII-BanII deletion was used as a vector for the synthetic oligonucleotides RW152 (SEQ ID NO:56) and RW153 (SEQ ID NO:57). These oligonucleotides 35 represent the 3' portion of the H6 promoter from the EcoRV site and align the ATG of the promoter with the ATG of the H4 cDNA.

RW152 (SEQ ID NO:56): 5' GCACGGAACAAAGCTTATCGCGATATCCGTTA
AGTTTGATCGTAATGCTATCAATCACGATTCTGTTCC
TGCTCATAGCAGAGGGCTCATCTCAGAAT 3'

RW153 (SEQ ID NO:57): 5' ATTCTGAGATGAGCCCTCTGCTATGAGCAGGA
5 ACAGAACATCGTATTGATAGCATTACGATACAAACTTA
ACGGATATCGCGATAAGCTTGTTCGTGC 3'

The oligonucleotides were annealed, cut with BanII and HindIII and inserted into the HindIII-BanII deleted pRW828 vector described above. The resulting plasmid 10 pRW844 was cut with EcoRV and DraI and the 1.7 kbp fragment containing the 3' H6 promoted H4 coding sequence was inserted between the EcoRV and HincII sites of pRW846 (described previously) forming plasmid pRW848. Plasmid pRW848 therefore contains the H4 coding sequence linked 15 to the vaccinia virus H6 promoter in the de-ORFed F8 locus of fowlpox virus.

Construction of Insertion Plasmid for H5

Hemagglutinin at the F8 Locus. A cDNA clone of avian influenza H5 derived from A/Turkey/Ireland/1378/83 was 20 received in plasmid pTH29 from Dr. R. Webster. Synthetic oligonucleotides RW10 (SEQ ID NO:58) through RW13 (SEQ ID NO:61) were designed to overlap the translation initiation codon of the previously described vaccinia virus H6 promoter with the ATG of the H5 gene. The 25 sequence continues through the 5' SalI site of the H5 gene and begins again at the 3' H5 DraI site containing the H5 stop codon.

RW10 (SEQ ID NO:58): 5' GAAAAATTAAAGTCGACCTGTTTGAGT
TGTGCGTGGTAACCAATGCAAATCTGGTC
30 ACT 3'
RW11 (SEQ ID NO:59): 5' TCTAGCAAGACTGACTATTGCAAAAAGAAGCA
CTATTCCTCCATTACGATACAAACTTAACG
GAT 3'
RW12 (SEQ ID NO:60): 5' ATCCGTTAAGTTGTATCGTAATGGAGGAAA
TAGTGCTTCTTTGCAATAGTCAGTCTTGCTAGAAGT
35 GACCAGATTGCATTGGT 3'

RW13 (SEQ ID NO:61): 5' TACCACGCAAACAACCTAACACAAACAGGTCG
ACTTTAAATTTCTGCA 3'

The oligonucleotides were annealed at 95°C for three minutes followed by slow cooling at room temperature.

5 This results in the following double strand structure with the indicated ends.



10



Cloning of oligonucleotides between the EcoRV and 15 PstI sites of pRW742B resulted in pRW744. Plasmid pRW742B contains the vaccinia virus H6 promoter linked to a non-pertinent gene inserted at the HincII site of pRW731.15 described previously. Digestion with PstI and EcoRV eliminates the non-pertinent gene and the 3'-end of 20 the H6 promoter. Plasmid pRW744 now contains the 3' portion of the H6 promoter overlapping the ATG of avian influenza H5. The plasmid also contains the H5 sequence through the 5' SalI site and the 3' sequence from the H5 stop codon (containing a DraI site). Use of the DraI 25 site removes the H5 3' non-coding end. The oligonucleotides add a transcription termination signal recognized by early vaccinia virus RNA polymerase (Yuen et al., 1987). To complete the H6 promoted H5 construct, the H5 coding region was isolated as a 1.6 kpb SalI-DraI 30 fragment from pTH29. Plasmid pRW744 was partially digested with DraI, the linear fragment isolated, recut with SalI and the plasmid now with eight bases deleted between SalI and DraI was used as a vector for the 1.6 kpb pTH29 SalI and DraI fragment. The resulting plasmid 35 pRW759 was cut with EcoRV and DraI. The 1.7 kbp pRW759 EcoRV-DraI fragment containing the 3' H6 promoter and the H5 gene was inserted between the EcoRV and HincII sites of pRW846 (previously described). The resulting plasmid pRW849 contains the H6 promoted avian influenza virus H5

gene in the de-ORFed F8 locus.

Construction of Insertion Vector for H7

Hemagglutinin at the F7 Locus. Plasmid pCVH71 containing the H7 hemagglutinin from A/CK/VIC/1/85 was received from 5 Dr. R. Webster. An EcoRI-BamHI fragment containing the H7 gene was blunt-ended with the Klenow fragment of DNA polymerase and inserted into the HincII site of pIBI25 as PRW827. Synthetic oligonucleotides RW165 (SEQ ID NO:62) and RW166 (SEQ ID NO:63) were annealed, cut with HincII 10 and StyI and inserted between the EcoRV and StyI sites of pRW827 to generate pRW845.

RW165 (SEQ ID NO:62): 5' GTACAGGTCGACAAGCTTCCCAGGGTATCGCG
ATATCCGTTAAGTTGTATCGTAATGAATACTCAAATT
CTAATACTCACTCTTGTGGCAGCCATTACACACAAATG
15 CAGACAAAATCTGCCTTGGACATCAT 3'

RW166 (SEQ ID NO:63): 5' ATGATGTCCAAGGCAGATTTGTCTGCATTG
TGTGAATGGCTGCCACAAGAGTGAGTATTAGAATTG
AGTATTTCATTACGATACAAACTAACGGATATCGCGA
TACCCGGGAAGCTTGTGACCTGTAC 3'

20 Oligonucleotides RW165 (SEQ ID NO:62) and RW166 (SEQ ID NO:63) link the 3' portion of the H6 promoter to the H7 gene. The 3' non-coding end of the H7 gene was removed by isolating the linear product of an ApaLI digestion of pRW845, recutting it with EcoRI, isolating 25 the largest fragment and annealing with synthetic oligonucleotides RW227 (SEQ ID NO:64) and RW228 (SEQ ID NO:65). The resulting plasmid was pRW854.

RW227 (SEQ ID NO:64): 5' ATAACATGCAGGTGCACCATTGTATAT
AAGTTAACGAATTCCAAGTCAAGC 3'

30 RW228 (SEQ ID NO:65): 5' GCTGACTTGGAAATTCTGTTAACTTATA
TACAAATGGTGCACCGCATGTTAT 3'

The stop codon of H7 in PRW854 is followed by an HpaI site. The intermediate H6 promoted H7 construct in the de-ORFed F7 locus (described below) was generated by 35 moving the pRW854 EcoRV-HpaI fragment into pRW858 which had been cut with EcoRV and blunt-ended at its PstI site.

Plasmid pRW858 (described below) contains the H6 promoter in an F7 de-ORFed insertion plasmid.

The plasmid pRW858 was constructed by insertion of an 850 bp SmaI/HpaI fragment, containing the H6 promoter 5 linked to a non-pertinent gene, into the SmaI site of pF7DO described previously. The non-pertinent sequences were excised by digestion of pRW858 with EcoRV (site 24 bp upstream of the 3'-end of the H6 promoter) and PstI. The 3.5 kb resultant fragment was isolated and blunt-10 ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. This blunt-ended fragment was ligated to a 1700 bp EcoRV/HpaI fragment derived from pRW854 (described previously). This EcoRV/HpaI fragment contains the entire AIV HA (H7) 15 gene juxtaposed 3' to the 3'-most 24 bp of the VV H6 promoter. The resultant plasmid was designated pRW861.

The 126 bp EH arm (defined previously) was lengthened in pRW861 to increase the recombination frequency with genomic TROVAC DNA. To accomplish this, a 20 575 bp AccI/SnaBI fragment was derived from pRW 731.13 (defined previously). The fragment was isolated and inserted between the AccI and NaeI sites of pRW861. The resultant plasmid, containing an EH arm of 725 bp and a HB arm of 404 bp flanking the AIV H7 gene, was designated 25 as pRW869. Plasmid pRW869 therefore consists of the H7 coding sequence linked at its 5' end to the vaccinia virus H6 promoter. The left flanking arm consists of 404 bp of TROVAC sequence and the right flanking arm of 725 bp of TROVAC sequence which directs insertion to the de-30 ORFed F7 locus.

Development of TROVAC-Avian Influenza Virus Recombinants. Insertion plasmids containing the avian influenza virus HA coding sequences were individually transfected into TROVAC infected primary CEF cells by 35 using the calcium phosphate precipitation method previously described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis

of hybridization to HA specific radiolabelled probes and subjected to sequential rounds of plaque purification until a pure population was achieved. One representative plaque was then amplified to produce a stock virus.

5 Plasmid pRW849 was used in an *in vitro* recombination test to produce recombinant TROVAC-AIH5 (vFP89) expressing the H5 hemagglutinin. Plasmid pRW848 was used to produce recombinant TROVAC-AIH4 (vFP92) expressing the H4 hemagglutinin. Plasmid pRW869 was used to produce
10 recombinant TROVAC-AIH7 (vFP100) expressing the H7 hemagglutinin.

Immunofluorescence. In influenza virus infected cells, the HA molecule is synthesized and glycosylated as a precursor molecule at the rough endoplasmic reticulum.

15 During passage to the plasma membrane it undergoes extensive post-translational modification culminating in proteolytic cleavage into the disulphide linked HA₁ and HA₂ subunits and insertion into the host cell membrane where it is subsequently incorporated into mature viral
20 envelopes. To determine whether the HA molecules produced in cells infected with the TROVAC-AIV recombinant viruses were expressed on the cell surface, immunofluorescence studies were performed. Indirect immunofluorescence was performed as described (Taylor et al., 1990). Surface expression of the H5 hemagglutinin in TROVAC-AIH5, H4 hemagglutinin in TROVAC-AIH4 and H7 hemagglutinin in TROVAC-AIH7 was confirmed by indirect immunofluorescence. Expression of the H5 hemagglutinin was detected using a pool of monoclonal antibodies
25 specific for the H5HA. Expression of the H4HA was analyzed using a goat monospecific anti-H4 serum. Expression of the H7HA was analyzed using a H7 specific monoclonal antibody preparation.

Immunoprecipitation. It has been determined that
30 the sequence at and around the cleavage site of the hemagglutinin molecule plays an important role in determining viral virulence since cleavage of the

hemagglutinin polypeptide is necessary for virus particles to be infectious. The hemagglutinin proteins of the virulent H5 and H7 viruses possess more than one basic amino acid at the carboxy terminus of HA1. It is 5 thought that this allows cellular proteases which recognize a series of basic amino acids to cleave the hemagglutinin and allow the infectious virus to spread both *in vitro* and *in vivo*. The hemagglutinin molecules of H4 avirulent strains are not cleaved in tissue culture 10 unless exogenous trypsin is added.

In order to determine that the hemagglutinin molecules expressed by the TROVAC recombinants were authentically processed, immunoprecipitation experiments were performed as described (Taylor et al., 1990) using 15 the specific reagents described above.

Immunoprecipitation analysis of the H5 hemagglutinin expressed by TROVAC-AIH5 (vFP89) showed that the glycoprotein is evident as the two cleavage products HA₁ and HA₂ with approximate molecular weights of 44 and 23 20 kDa, respectively. No such proteins were precipitated from uninfected cells or cells infected with parental TROVAC. Similarly immunoprecipitation analysis of the hemagglutinin expressed by TROVAC-AIH7 (vFP100) showed specific precipitation of the HA₂ cleavage product. The 25 HA₁ cleavage product was not recognized. No proteins were specifically precipitated from uninfected CEF cells or TROVAC infected CEF cells. In contrast, immunoprecipitation analysis of the expression product of TROVAC-AIH4 (vFP92) showed expression of only the 30 precursor protein HA₀. This is in agreement with the lack of cleavage of the hemagglutinins of avirulent subtypes in tissue culture. No H4 specific proteins were detected in uninfected CEF cells or cells infected with TROVAC. Generation of recombinant virus by 35 recombination, *in situ* hybridization of nitrocellulose filters and screening for B-galactosidase activity are as previously described (Panicali et al., 1982; Perkus et

al., 1989).

Example 14 - GENERATION OF NYVAC- AND ALVAC-BASED RECOMBINANT CONTAINING THE GENE ENCODING HUMAN TUMOR NECROSIS FACTOR - α (TNF- α)

5 TNF- α is a cytokine produced by CTLs. It is one of the products of these cells that is responsible for killing tumor cells during an immune response. It has previously been shown that the injection of recombinant
10 TNF could mediate the necrosis and regression of a variety of established murine cancers (Asher et al., 1987). The exact mechanisms for this anti-tumor activity remain unclear, although TNF apparently affects the vascular supply of tumors (Asher et al., 1987). Both the
15 secreted and membrane-bound forms of TNF- α may be critical for its anti-tumor activities (Kriegler et al., 1987).

Plasmid pE4, containing the human necrosis factor - α gene was extracted from *E. coli* transformed with this
20 plasmid. The pE4 transformed *E. coli* were obtained from ATCC (ATCC #CLN-39894). PCR fragment PCR-TNF4 (755 bp) was generated using pE4 as template and oligonucleotides TNF3 (SEQ ID NO:66); 5'-
ATCATCTCGCGATATCCGTTAAGTTGTATCGTAATGAGCACTGAAAGCATGATC-
25 3') containing the 3'-most region of the vaccinia H6 promoter (from the NruI site to the end; Perkus et al., 1989) and the first 21 bp of the TNF- α coding sequence, and oligonucleotide TNF2 (SEQ ID NO:67) (5'-
ATCATCTCTAGAATAAAATCACAGGGCAATGATCCC-3'), containing the
30 last 15 bp of the TNF- α coding sequence, a vaccinia early transcription termination signal (T₅NT; Yuen and Moss, 1986), and an XbaI restriction site. A complete NruI/XbaI digestion was performed and the resultant 735 bp fragment was isolated and inserted into the 4.8 kb
35 NruI/XbaI fragment obtained by the digestion of the generic insertion plasmid pVQH6C5LSP. The resultant plasmid was designated pMAW048. The nucleotide sequence of the entire H6-TNF- α expression cassette was confirmed as described by Goebel et al. (1990).

Plasmid pVQH6C5LSP was derived in the following manner:

A C5 insertion vector containing 1535 bp upstream of C5, polylinker containing KpnI, SmaI, XbaI, and NotI sites, and 404 bp of canarypox DNA (31 bp of C5 coding sequence and 373 bp of downstream sequence) was derived in the following manner. A genomic library of canarypox DNA was constructed in the cosmid vector pVK102 (Knauf et al., 1982) probed with pRW764.5 and a clone containing a 29 kb insert identified (pHCOS1). A 3.3 kb ClaI fragment from pHCOS1 containing the C5 region was identified. Sequence analysis of the ClaI fragment was used to extend the sequence in FIG. 8 (SEQ ID NO:68) from nucleotides 1-1372.

The C5 insertion vector was constructed as follows. The 1535 bp upstream sequence was generated by PCR amplification using oligonucleotides C5A (SEQ ID NO:69) (5'-ATCATCGAATTCTGAATGTTAAATGTTATACCTTG-3') and C5B (SEQ ID NO:75) (5'-GGGGGTACCTTGAGAGTACCACTTCAG-3') and purified genomic canarypox DNA as template. This fragment was digested with EcoRI (within oligo C5A) and cloned into EcoRI/SmaI digested pUC8 generating pC5LAB. The 404 bp arm was generated by PCR amplification using oligonucleotides C5C (SEQ ID NO:71) (5'-GGGTCTAGAGCGGCCGTTATAAAGATCTAAAATGCATAATTC-3') and C5DA (SEQ ID NO:72) (5'-ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3'). This fragment was digested with PstI (within oligo C5DA) and cloned into SmaI/PstI digested pC5LAB generating pC5L.

pC5L was digested within the polylinker with Asp718 and NotI, treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides CP26 (SEQ ID NO:73) (5'-GTACGTGACTAATTAGCTATAAAAGGATCCGGTACCCCTCGAGTCTAGAATCG ATCCCCGGTTTTATGACTAGTTAACAC-3') and CP27 (SEQ ID NO:74) (5'-GCCCGTGATTAACTAGTCATAAAACCCGGGATCGATTCTAGACTCGAGGGTA CGGGATCCTTTATAGCTAATTAGTCAC-3') (containing a disabled

Asp718 site, translation stop codons in six reading frames, vaccinia early transcription termination signal (Yuen and Moss, 1987), BamHI, KpnI, XhoI, XbaI, and SmaI restriction sites, vaccinia early transcription 5 termination signal, translation stop codons in six reading frames, and a disabled NotI site) generating plasmid pC5LSP.

The early/late H6 vaccinia virus promoter (Perkus et al., 1989) was derived by PCR from a plasmid containing 10 the promoter using oligonucleotides CP30 (SEQ ID NO:75) (5'-TCGGGATCCGGGTTAATTAGTCATCAGGCAGGGCG-3') and CP31 (SEQ ID NO:76) (5'-TAGCTCGAGGGTACCTACGATAACAAACTAACGGATATCG-3'). The PCR product was digested with BamHI and XhoI (sites 15 created at the 5' and 3' termini by the PCR) and ligated to similarly digested pC5LSP generating pVQH6C5LSP.

Plasmid pMAW048 was used in *in vitro* recombination assays with ALVAC (CP_{ppp}) as rescue virus to yield recombinant virus vCP245. Insertion with this plasmid 20 replaces the two copies of the C5 open reading frame with the human TNF- α expression cassette. Fig. 15 presents the nucleotide sequence of the H6/TNF- α sequence and flanking regions within vCP245 (SEQ ID NO:79). The H6 promoter starts at position 74. The TNF- α start codon is 25 at position 201, and the TNF- α stop codon is at position 902. Positions 1 through 73 and positions 903 through 965 flank the H6/TNF- α expression cassette.

PCR fragment PCR-TNFH6 (156 bp) was amplified from 30 plasmid pBSH6 using oligonucleotides H65PH (SEQ ID NO:80) (5'-ATCATCAAGCTTGATTCTTATTCTATAC-3') containing a HindIII site in the initial 21 bp of the H6 promoter region and TNFH6 (SEQ ID NO:81) (5'-CATGCTTCAGTGCTCATTACGATAACAAACTAACGG-3') containing the 3'-most 19 nucleotides of the H6 promoter and the 5'-most 35 18 nucleotides of the TNF coding sequence.

Plasmid pBSH6 was generated in the following manner. The vaccinia H6 promoter through the EcoRV site was

derived from a plasmid containing the synthetic H6 promoter (Perkus et al., 1989) using PCR and primers H6PCR2 (SEQ ID NO:82) (5'-TTAACGGATATCGCGATAATG-3') and H6PCR1 (SEQ ID NO:83) (5'-
5 ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG-3') creating a 5'
10 HindIII site. This 122 bp PCR-derived fragment was digested with HindIII and EcoRV followed by ligation to similarly digested pBS-SK⁺ (Stratagene, La Jolla, CA) generating plasmid pBSH6. The insert was confirmed by nucleotide sequence analysis.

PCR fragment PCR-TNF (721 bp) was amplified from plasmid pE4 using oligonucleotides TNF1 (SEQ ID NO:84) (5'-ATGAGCACTGAAAGCATG-3') containing the initial 18 nucleotides of the TNF- α coding sequence and TNF2 (SEQ ID NO:67). The PCR fragment, PCR-TNF fusion (859 bp), was generated using PCR-TNFH6 and PCR-TNF as templates and oligonucleotides H65PH (SEQ ID NO:80) and TNF2 (SEQ ID NO:67) as primers. PCR-TNF fusion was digested with HindIII and XbaI and the resultant 841 bp fragment was inserted into pBS-SK⁺ (Stratagene, La Jolla, CA) digested with HindIII and XbaI. The resultant plasmid was designated pMAW047 and the H6-TNF cassette was confirmed by nucleotide sequence analysis as described previously (Goebel et al., 1990).

25 The 841 bp HindIII/XbaI fragment containing the H6-TNF- α expression cassette was isolated from pMAW047, blunt-ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs, and inserted into the vaccinia insertion plasmid pSD541. The 30 resultant plasmid was designated pMAW049.

Plasmid pSD541 was derived in the following manner. Flanking arms for the ATI region were generated by PCR using subclones of the Copenhagen HindIII A region as

template. Oligonucleotides MPSYN267 (SEQ ID NO:85) (5'-GGGCTCAAGCTTGCAGGCCGCTCATTAGACAAGCGAATGAGGGAC-3') and MPSYN268 (SEQ ID NO:86) (5'-AGATCTCCCAGGGCTCGAGTAATTAATTAATTTTATTACACCAGAAAAGACGGCTTG-3') were used to derive the 420 bp vaccinia arm to the right of the ATI deletion. Synthetic oligonucleotides MPSYN269 (SEQ ID NO:87) (5'-TAATTACTCGAGCCCGGGAGATCTAATTAATTTATATAACTCATTTTG-3') and MPSYN270 (SEQ ID NO:88) (5'-ATATACT-3') and MPSYN270 (SEQ ID NO:88) (5'-TATCTCGAATTCCCGCGGCTTAAATGGACGGAACCTCTTTCCCC-3') were used to derive the 420 bp vaccinia arm to the left of the deletion. The left and right arms were fused together by PCR and are separated by a polylinker region specifying restriction sites for BglII, SmaI, and XhoI. The PCR-generated fragment was digested with HindIII and EcoRI to yield sticky ends, and ligated into pUC8 digested with HindIII and EcoRI to generate pSD541.

The plasmid pMAW047 was used in *in vitro* recombination assays (Piccini et al., 1987) with NYVAC (vP866; Tartaglia et al., 1992) as the rescue virus. Recombination with this plasmid replaces the ATI open reading frame with the H6-TNF- α expression cassette. The NYVAC recombinant virus containing the H6-TNF- α cassette was designated vP1200. Fig. 16 presents the nucleotide sequence of the H6/TNF- α expression cassette incorporated into the NYVAC recombinant, vP1200, and flanking NYVAC sequences (SEQ ID NO:89). The H6 promoter starts at position 59. The TNF- α start codon is at position 185, and the TNF- α stop codon is at position 884. Positions 1 through 58 and positions 885 through 947 flank the H6/TNF- α expression cassette.

Table 21. Expression of Human TNF- α by vP1200 and vCP245

Sample	Description	TNF- α (ng/ml)
5 vP1196	NYVAC-CMVgB+pp65	0
vP1200	NYVAC-TNF- α	>240
CPpp	ALVAC	0
vCP245	ALVAC-TNF- α	59

10 Expression of TNF- α by vP1200 (NYVAC-TNF- α) and vCP245
 (ALVAC-TNF- α) was measured by ELISA assay, using a
 commercially available kit (Genzyme Diagnostics,
 Cambridge, MA, cat.#1915-01). Samples were prepared by
 infection of Vero cells (NYVAC recombinants) or primary
 15 chick embryo fibroblasts (ALVAC) with recombinant or
 parent virus. The cells were harvested when CPE was
 complete and the infected cell lysates were used for the
 ELISA assay, after sonication and clarification by
 centrifugation at 500 xg for 10 min. One control,
 20 vP1196, which expresses two cytomegalovirus proteins, gB
 and pp65, was prepared in the same manner as the TNF- α
 recombinants. The other control, ALVAC parent, was a
 partially purified virus stock. All samples contained
 approximately 10^7 PFU/ml of virus. The results, shown in
 25 Table 21, indicate that both vP1200 and vCP245 are
 expressing human TNF- α . Expression of such levels *in*
vivo can be therapeutic.

Example 15 - NYVAC AND ALVAC-BASED p53 RECOMBINANT VIRUSES

30 The nuclear phosphoprotein, p53, is found in normal
 cells at very low steady state levels. Expression of p53
 is tightly regulated throughout the cell cycle and may be
 involved in controlling cell proliferation. The
 35 molecular mechanisms by which p53 exerts its tumor
 suppressor activity remain unknown, although p53 appears
 to exist in two conformational states. One form is
 unique to wildtype p53 and is associated with the ability

to block cell cycle progression while the second form is associated with the ability to promote cell proliferation and is common to wildtype and mutant forms (reviewed by Ulrich et al., 1992). p53 is the gene most frequently 5 found to be mutated in a wide variety of human tumors (reviewed by Hollstein et al., 1991).

Probably the most studied cancer associated with p53 mutation is breast cancer. It is known that p53 mutation results in the overexpression of the p53 gene product in 10 primary breast cancer patients (Davidoff et al., 1991). The basis for p53 overexpression was found to result from a post-transcriptional mechanism, since p53-specific mRNA levels were similar in tumors with high and low level protein expression. Further, the p53 mRNA from 15 overexpressing tumors were found to contain missense mutations in highly conserved regions of the gene. These mutations were subsequently found to give rise to more stable p53 protein forms which form complexes with heat shock protein 70 (HSP-70). Since HSP-70 proteins have 20 been implicated in antigen processing, not only may the humoral response to p53 observed in a subset of breast cancer patients have resulted from unique processing/presentation modes for complexes, such an association may also elicit cellular anti-p53 protein 25 responses (Davidoff et al., 1992). Such anti-p53 cellular immune responses may be more germane to the eventual immunotherapy of such cancers.

Generation of Poxvirus-based Recombinant Viruses

Expressing Wildtype and Mutant Forms of the Human p53

30 Gene Product

Three plasmids, p53wtXbaISP6/T3, p53-217XbaI, and p53-238XbaI containing wildtype human p53 gene sequences, and two mutant forms of p53, respectively, were obtained from Dr. Jeffrey Marks (Duke University). The p53- 35 217XbaI contains a p53 gene encoding a p53 product lacking codon 217 while p53-238XbaI encodes a p53 gene product with an cysteine to arginine substitution at

amino acid 238. The sequence of the wildtype p53 cDNA and the deduced amino acid sequence was described previously (Lamb and Crawford, 1986; FIG. 3).

All three p53 genes were individually juxtaposed 3' to the modified vaccinia virus H6 promoter described by Perkus et al., 1989. These manipulations were performed in the following manner. A 227 bp PCR-derived fragment was generated using oligonucleotides MM002 (SEQ ID NO:90) (5'-GATCTGACTGCGCTCCTCCATTACGATAACAAACTTAACGG-3') and 10 RW425 (SEQ ID NO:91) (5'- GTGGGTAAGGAATTCGGATCCCCGGGTTAATTAAATTAGTGATAC-3') and plasmid pRW825 as template. PCR using these oligonucleotides amplifies the vaccinia H6 promoter sequences from pRW825 such that the 3' end of the 15 promoter is precisely linked to the 5'-most region of the p53 coding sequence. Plasmid pRW825 contains the vaccinia virus H6 promoter (Perkus et al., 1989) linked to a nonpertinent gene.

PCR was also used to generate a 480 bp and 250 bp fragment from p53wtXbaISP6/T3. The 480 bp fragment was 20 derived with oligonucleotides MM003 (SEQ ID NO:92) (5'- GTTTGTATCGTAATGGAGGGAGCCGCAGTCAGATC-3') and MM008 (SEQ ID NO:93) (5'- CATTACGATAACAAACTTAACGGATATCGCGACCGCGTTCACACAGGGCAGGTCTGGC 25 -3'). This fragment contains the 3' portion of the vaccinia virus H6 promoter sequences and the 5' portion of the p53 coding sequences through the SgrAI site. The 250 bp fragment was derived by amplification with oligonucleotides MM005 (SEQ ID NO:94) (5'- 30 TACTACCTCGAGCCCAGGATAAAAAACCGCGTTCACTGAGTCAGGCC-3') and MM007 (SEQ ID NO:95) (5'- GTGTGAAACCGCGTCGCGATATCCGTTAAGTTGTATCGTAATGCAGCTGCGTGGCGT 35 GAGCGCTTC-3'). This PCR fragment contains the 3' end of the p53 coding sequences beginning at the StuI restriction site. The 480 bp and 250 bp PCR fragments were generated such that the 5' end of the MM005/MM007-derived (SEQ ID NO:94/95) fragment overlaps the 3' end of

the MM003/MM008-derived (SEQ ID NO:92/93) fragment.

The 227 bp, 480 bp, and 250 bp PCR-derived fragments were pooled and fused by PCR using oligonucleotides MM006 (SEQ ID NO:96) (5'-ATCATCGGATCCCCGGGTTCTTATTCTATAC-3') and MM005 (SEQ ID NO:94). The 783 bp fused PCR product contains the H6 promoter juxtaposed 5' to the 5' portion of the p53 coding sequence (through the SgrAI restriction site) followed by the end of the p53 coding sequence beginning at the StuI site. Following the end of the p53 10 coding sequence, a T₅NT sequence motif providing early vaccinia transcription termination (Yuen and Moss, 1986) and a unique XhoI site were added. It should be noted that the final H6-p53 PCR fusion product (783 bp) does not contain the p53 coding sequences between the SgrAI 15 and StuI restriction sites.

The 783 bp fusion was digested with BamHI (5' end) and XhoI (3' end) and inserted into plasmid pSD550 to yield plasmid pMM105. Plasmid pSD550 enables the insertion of foreign genes into the vaccinia I4L locus by 20 replacing the I4L coding sequence. This plasmid was derived from pSD548 (Tartaglia et al., 1992) by first digesting this plasmid with BglII and SmaI. This digested plasmid was then ligated to annealed oligonucleotides 539A (SEQ ID NO:97) (5'- 25 AGAAAAATCAGTTAGCTAAGATCTCCGGGCTCGAGGGTACCGGATCCTGATTAGTT AATTTTTGT-3') and 539B (SEQ ID NO:98) (5'- GATCACAAAAATTAACTAATCAGGATCCGGTACCCTCGAGCCCGGGAGATCTTAGCT AACTGATTTTCT-3) to generate pSD550.

Plasmids containing intact p53 gene (wildtype or 30 mutant forms) juxtaposed 3' to the H6 promoter were generated by first digesting pMM105 with SgrAI and StuI. A 795 bp SgrAI/StuI fragment was isolated from p53wtXbaISP6/T3 and p53-238XbaI, while a 792 bp fragment was isolated from p53-217XbaI. These fragments were 35 individually ligated to the SgrAI/StuI digested pMM105 plasmid to yield pMM106, pMM108, and pMM107, respectively.

Plasmids pMM106, pMM107, and pMM108 were used in standard *in vitro* recombination experiments (Piccini et al., 1987) with NYVAC (vP866; Tartaglia et al., 1992) as the rescue virus to generate recombinant viruses vP1101, 5 vP1096, and vP1098, respectively. Fig. 17 presents the nucleotide sequence of the wildtype p53 expression cassette and flanking regions within vP1101 (SEQ ID NO:99). The H6 promoter starts at position 145. The p53 start codon is at position 269, and the p53 stop codon is 10 at position 1450. Positions 1 through 144 and positions 1451 through 1512 flank the H6/p53 expression cassette. The sequences within vP1096 and vP1098 are identical except vP1096 contains a 3 base deletion from nucleotide 920 to 922 while vP1101 contains a point mutation at 15 nucleotide 980 (T or C).

Both immunofluorescence and immunoprecipitation assays were performed using a p53-specific monoclonal antibody (pAB1801, Oncogene Science provided by Dr. J. Marks) to demonstrate expression of p53 in vP1101, vP1098 20 and vP1096 infected Vero cells. These assays were performed as described previously (Taylor et al., 1990). Immunofluorescence assay demonstrated p53-specific fluorescent staining of cells infected with vP1101, vP1096, or vP1098. The p53 antigen was located in both 25 the nucleus and cytoplasm of the infected cells. The nuclear staining, however, was more intense in vP1101 infected cells. These results are similar to those reported by Ronen et al. (1992) using replication-competent vaccinia to express wildtype and mutant forms 30 of p53. No p53-specific fluorescent staining was observed in Vero cells infected with the parental NYVAC virus, vP866.

ALVAC (CPpp) p53 insertion plasmids were engineered by excising the p53 expression cassettes from pMM106, 35 pMM107, and pMM108 by digestion with BamHI and XhoI and inserting them individually into BamHI/XhoI digested pNVQC5LSP-7. The 1320 bp BamHI/XhoI fragment containing

the H6-p53 expression cassette from pMM106 and pMM108 was inserted into pNVQC5LSP-7 to yield pMM110 and pMM112, respectively, while the 1317 bp BamHI/XbaI fragment derived from pMM107 and inserted into pNVQC5LSP-7 yielded pMM111.

5 The plasmid pNVQC5LSP-7 was derived in the following manner. pc5LSP (defined in Example 1) was digested with BamHI and ligated to annealed oligonucleotides CP32 (SEQ ID NO:100) (5'-

10 CATCTTAATTAAATTAGTCATCAGGCAGGGCGAGAACGAAAGACTATCTGCTCGTTAAT TAATTAGGTGACG-3') and CP33 (SEQ ID NO:101) (5'- CATCCGTCGACCTAATTAAACGACGACATAGTCTCGTTCTGCCCTGCCTGATGA CTAATTAAATTAA-3') to generate pVQC5LSP6. pVQC5LSP6 was digested with EcoRI, treated with alkaline phosphatase 15 and ligated to self-annealed kinased oligonucleotide CP29 (SEQ ID NO:102) (5'-AATTGCGGCCGC-3'), digested with NotI and linear was purified followed by self-ligation. This procedure introduced a NotI site to pVQC5LSP6, generating pNVQC5LSP-7.

20 Insertion plasmids pMM110, pMM111, and pMM112 were used in standard in vitro recombination experiments (Piccini et al., 1987) with ALVAC (CPpp) as the rescue virus to yield vCP207, vCP193 and vCP191, respectively. Confirmation of expression of the p53 gene product was 25 accomplished by immunoprecipitation assays performed as described above. Fig. 18 presents the nucleotide sequence of the H6/p53 (wildtype) expression cassette and flanking regions from vCP207 (SEQ ID NO:103). The H6 promoter starts at position 109. The p53 start codon is 30 at position 233, and the p53 stop codon is at position 1414. Positions 1 through 232 and positions 1415 through 1483 flank the H6/p53 expression cassette. The nucleotide sequence is identical to that within vCP193 and vCP191 except vCP193 contains a 3 nucleotide deletion 35 from nucleotide 973 to 975 while vCP191 contains a point mutation at nucleotide 94 to (T to C).

A listing of the NYVAC- and ALVAC- based p53

recombinant viruses is provided in Table 22.

TABLE 22. NYVAC and ALVAC-based p53 recombinant viruses

	<u>Recombinant Virus</u>	<u>Parent Virus</u>	<u>Gene Insert</u>
5	vP1101	NYVAC	w.t. 53
	vP1096	NYVAC	p53(-aa 217)
	vP1098	NYVAC	p53 (aa238; C to R)
	vCP207	ALVAC	w.t. 53
	vCP193	ALVAC	p53 (-aa 217)
10	vCP191	ALVAC	p53 (aa 238; C to R)

Example 16 - UTILITY OF NYVAC- AND ALVAC-BASED RECOMBINANT VIRUSES CONTAINING THE MAGE-1 GENE

15 Human melanoma-associated antigen MZ2-E is encoded by the MAGE-1 gene (Reviewed by van der Bruggen and Van der Eynde, 1992). MAGE-1 is expressed in primary melanoma tumor cells, melanoma-derived cell lines, and 20 certain tumors of non-melanoma origins but not in normal cells except in testis (Coulie et al., 1993). Of interest from an immunological perspective, CTLs from melanoma-bearing patients that are of the HLA-A1 MHC haplotype are known to recognize a nonapeptide from the 25 MZ2-E gene product (Traveseri et al., 1992). Therefore, definition of such an antigen provides a mechanism for targeted immunotherapy for HLA-typed (HLA-A1) melanoma patients.

Generation of NYVAC- and ALVAC-based Recombinant Viruses Containing the MAGE-1 Gene

30 PCR fragment PCR-H6 (162 bp) was synthesized using pBSH6 (described in Example 14) as template and oligonucleotides H65PH (SEQ ID NO:80) and M1-4 (SEQ ID NO:104) (5'-CAGACTCCTCTGCTCAAGAGACATTACGATAACAACTTAACG-3') which contains the last 18 bp of the H6 promoter and the initial 24 nucleotides of the MAGE-1 gene. A second 35 PCR fragment (PCR-M1) was amplified from plasmid pTZ18RMAGE1 using oligonucleotides M1-1 (SEQ ID NO:105)

(5'-ATGTCTCTGAGCAGAGGAGTCTG-3' and M1-2 (SEQ ID NO:106) (5'-CAGGCCATCATAGGAGAGACC-3'). The resultant PCR fragment represents the initial 546 bp of the MAGE-1 coding sequence.

5 Plasmid pTZ18RMAGE-1 contains a cDNA clone of the MAGE-1 gene. This gene encodes the MZE-2 human melanoma rejection antigen. This plasmid was provided by Dr. Lloyd Old (Memorial Sloan-Kettering, NY, NY) who obtained the plasmid originally from Dr. Thierry Boon (Ludwig 10 Inst. for Cancer Research, Brussels, Belgium).

PCR fusion product, PCR-H6M1 was generated using PCR-H6 and PCR-M1 as templates and oligonucleotides H65PH (SEQ ID NO:80) and M1-2 (SEQ ID NO:106) as primers. A complete HindIII/BglII digestion of PCR-H6M1 was 15 performed and the resultant 556 bp was purified for subsequent cloning steps.

PCR fragment PCR-M3' (535 bp) was amplified from pTZ18RMAGE-1 using oligonucleotides M1-3 (SEQ ID NO:107) (5'-GTGGCTGATTGGTTGGTTCTG-3') which contains 24 20 nucleotides complementary to the MAGE-1 gene at a region approximately 200 bp upstream of the M1-2 oligonucleotide sequence and M1-5 (SEQ ID NO:108) (5'- ATCATCTCTAGAAAAAAATCACATAGCTGGTTTCAG-3') containing the terminal 15 nucleotides of the MAGE-1 coding sequence, a 25 vaccinia early transcription termination signal (T₅NT; Yuen and Moss, 1986) and an XbaI restriction site. PCR-M3' was digested with BglII and XbaI. The resultant 414 bp fragment was isolated and co-inserted into HindIII/XbaI digested pBS-SK(+) with the 556 bp 30 HindIII/BglII digested PCR fragment PCR-H6M1. The resultant plasmid containing the entire H6-MAGE-1 expression cassette was designated pMAW034. The H6-MAGE-1 cassette was confirmed by nucleotide sequence analysis as per Goebel et al., 1990).

35 The 864 bp NruI/XbaI fragment from pMAW034 was isolated and inserted into pVQH6C5LSP (described in Example 14) that was digested in a similar fashion. The

resultant plasmid was designated pMAW036. This plasmid served as the insertion plasmid for replacing the two C5 ORFs in the ALVAC genome with the H6-MAGE-1 expression cassette.

5 Plasmid pMAW036 was used in standard *in vitro* recombination experiments with ALVAC as the rescuing virus. Recombinant virus were identified by an *in situ* plaque hybridization assay using MAGE-1-specific radiolabeled DNA probes. Recombinant plaques were plaque 10 purified and amplified. The resultant ALVAC-based recombinant containing the MAGE-1 gene was designated vCP235. Fig. 19 presents the nucleotide sequence of the H6/MAGE-1 expression cassette and flanking region contained within vCP235 (SEQ ID NO:109). The H6 promoter 15 starts at position 74. The MAGE-1 start codon is at position 201, and the MAGE-1 stop codon is at position 1031. Positions 1 through 73 and positions 1032 through 1094 flank the H6/MAGE-1 expression cassette.

The NYVAC (vP866) insertion plasmid pMAW037 was 20 generated by initially digesting pMAW034 with NruI/BamHI. The resultant 879 bp fragment was isolated and inserted into NruI/BamHI digested pSPH46. The resultant plasmid was designated pMAW037.

Plasmid pSPH46 was generated in the following 25 manner. Plasmid pSD544 (containing vaccinia sequences surrounding the site of the HA gene replaced with a polylinker region and translation termination codons in six reading frames) was digested with XhoI within the polylinker, filled in with the Klenow fragment of DNA 30 polymerase I and treated with alkaline phosphatase. SP126 (containing the vaccinia H6 promoter) was digested with HindIII, treated with Klenow and the H6 promoter isolated by digestion with SmaI. Ligation of the H6 promoter fragment to pSD544 generated SPHA-H6 which 35 contained the H6 promoter in the polylinker region (in the direction of HA transcription).

Plasmid pMAW037 was used in standard *in vitro*

recombination experiments (Piccini et al., 1987) with NYVAC (vP866) as the rescue virus. Fig. 20 presents the nucleotide sequence of the H6/MAGE-1 expression cassette and flanking regions within pMAW037 (SEQ ID NO:110). The 5 H6 promoter starts at position 52. The MAGE-1 start codon is at position 179, and the MAGE-1 stop codon is at position 1009. Positions 1 through 51 and positions 1010 through 1084 flank the H6/MAGE-1 expression cassette.

10 **Example 17 - GENERATION OF AN ALVAC- AND NYVAC-BASED CEA RECOMBINANT VIRUSES**

The CEA gene was provided in plasmid pGEM.CEA, which contains the CEA coding sequence (2,109 nucleotides) as well as 5' and 3' untranslated regions (Dr. J. Schlom, 15 NCI-NIH). The 5' end of the CEA construct was modified to remove the 5' untranslated sequences and place the vaccinia H6 promotor before the ATG initiation codon of CEA. This was accomplished by PCR with the oligonucleotide pair CEA1 (SEQ ID NO:111) (5'- 20 TATCGCGATATCCGTTAACGTTGTATCGTAATGGAGTCTCCCTCG-3') and CEA2 (SEQ ID NO:112) (5'-TGCTAGATCTTATCTCTCGACCACTGTATG-3') and plasmid pGEM.CEA as template. The resulting fragment links the 3' 30 nucleotides of the H6 promotor to the CEA initiation codon, extends 22 nucleotides past 25 the ApaI site at position 278 of the CEA coding sequence, and terminates with a BglII site introduced by the PCR primer CEA2 (SEQ ID NO:114). Prior to cloning, this fragment was digested with EcoRV (site located within the 3' end of the H6 promotor) and BglII. The digested 5' 30 PCR fragment was then included in a 3-way ligation with two fragments derived from plasmid pI4L.H6: an NcoI/BglII vector fragment and an NcoI/EcoRV fragment which contained the 5' portion of the H6 promotor. The resulting plasmid, designated pI4L.H6.CEA-5', contains 35 the full length H6 promotor linked to a 5' CEA fragment extending from the ATG codon through the ApaI site at position 278.

The 3' end of CEA was modified to remove the 3'

untranslated region of CEA and place a vaccinia early transcription termination signal (T₅NT) followed by a series of restriction sites (XhoI, XbaI, SmaI, HindIII) after the TAG termination codon. This was accomplished 5 by PCR with the oligonucleotide pair CEA3 (SEQ ID NO:113) (5'-CTATGAGTGTGGAATCCAGAACG-3') and CEA4 (SEQ ID NO:114) (5'-TCAGAAGCTTCCCGGTCTAGACTCGAGATAAAACTATATCAGAGCAACC-3') and plasmid pGEM.CEA as template. The resulting fragment extends from a position 32 nucleotides 5' of the 10 CEA HindII site located at position 1203 through the 3' end of the coding sequence. This fragment was cloned as a HindII/HindIII fragment into a HindII/HindIII-digested pGEM.CEA vector fragment. The resulting plasmid, designated pGEM.CEA-3', contains the entire CEA gene as 15 found in pGEM.CEA with a 3' end modified to remove the 3' untranslated region and replace it with a T₅NT signal followed by XhoI, XbaI, SmaI, and HindIII restriction sites.

To generate an ALVAC C3 donor plasmid containing 20 CEA, a BamHI/ApaI fragment containing the H6 promotor linked to the 5' end of CEA was obtained from pI4L.H6.CEA-5', an ApaI/XhoI fragment containing the remainder of the CEA coding sequence (plus T₅NT) was obtained from pGEM.CEA-3', and a BamHI/XhoI C3 vector 25 fragment was derived from plasmid p126.C3. After subsequent 3-way ligation, the plasmid pH6.CEA.C3 was obtained. This plasmid contains the full length H6/CEA expression cassette inserted between left and right flanking arms of ALVAC DNA which direct insertion to the 30 C3 sites on the ALVAC genome. Transcription of CEA is oriented from right to left.

Plasmid p126.C3, an ALVAC C3 donor plasmid, was derived as follows. This plasmid contains an insert consisting of cDNA derived from the *Plasmodium falciparum* 35 SERA gene (Li et al., 1989; Bzik et al., 1989; Knapp et al., 1989; NOTE: SERA is also known as SERP I and p126) under the control of the entomopox virus 42K early

promotor.

A. Methodology for generating p126.C3.

1. Construction of *P. falciparum* FCR3 Strain Blood Stage cDNA Library.

5 Total RNA from human erythrocytes infected with *P. falciparum* FCR3 strain was provided by Dr. P. Delplace (INSERM-U42). Poly-A⁺ RNA was isolated from this sample by use of oligo(dT) cellulose (Stratagene, La Jolla, CA.) as described by Aviv and Leder (1972) and modified by
10 Kingston (1987). Briefly, total RNA was mixed with oligo(dT) cellulose in Binding buffer (0.5M NaCl, 0.01M Tris-Cl, pH 7.5) and incubated for 30 minutes at room temperature. Poly-A⁺ RNA/oligo(dT) cellulose complexes were pelleted by centrifugation and washed 3 times with
15 Binding buffer. Purified poly-A⁺ RNA was eluted from the oligo(dT) cellulose in Elution buffer (0.01M Tris-Cl, pH 7.5). A second elution with DEPC-treated dH₂O was performed, the eluates were pooled, and the poly-A⁺ RNA recovered by ethanol precipitation.

20 The purified poly-A⁺ RNA was used as a template for the synthesis of first strand cDNA by reverse transcriptase in a reaction primed with oligo(dT) (Watson and Jackson, 1985; Klickstein and Neve, 1987). For this reaction, 12ug poly-A⁺ RNA was incubated with 105 units
25 AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL.) in 100mM Tris-Cl pH 8.3, 30mM KCl, 6mM MgCl₂, 25mM DTT, 80 units RNasin, 1mM each dNTP, and 24ug/ml oligo(dT)₁₂₋₁₈ as primer for 2 hours at 42°C. After organic extractions, double stranded cDNA was
30 obtained by use of DNA polymerase I and RNase H with first strand cDNA as template (Watson and Jackson, 1985; Klickstein and Neve, 1987). The first strand cDNA was incubated with 25 units DNA polymerase I and 1 unit RNase H in 20mM Tris-Cl pH 6, 5mM MgCl₂, 10mM (NH₄)₂SO₄, 100mM
35 KCl, 500ug/ml BSA, 25mM DTT, and 0.1mM each dNTP at 12°C for one hour followed by one hour at room temperature to synthesize second strand cDNA. The double stranded cDNA

was recovered by organic extractions and ethanol precipitation.

The double-stranded blood stage cDNA was then sequentially treated with T4 DNA polymerase to create 5 blunt ends and EcoRI methylase to protect internal EcoRI sites. EcoRI linkers were then added followed by digestion with EcoRI and size selection on a 5-25% sucrose gradient. Fractions containing long cDNAs (1-10 Kb) were pooled and ligated into EcoRI cleaved Lambda 10 ZAPII vector (Stratagene, La Jolla, CA.). The resulting phage were packaged and used to infect the XL-1 Blue *E. coli* strain (Stratagene). The phage were then harvested from these cells and amplified by one additional cycle of 15 infection of XL-1 Blue to produce a high titer FCR3 strain blood stage cDNA library.

2. Screen of cDNA Library for SERA cDNA Clones.

The FCR3 strain cDNA library was screened by plaque hybridization with ³²P end-labelled oligonucleotides derived from published sequences of SERA to detect cDNA. 20 The cDNA library was plaqued on lawns of XL-1 Blue (Stratagene) in 150mm dishes at a density of 100,000 plaques per dish. Plaques were transferred to nitrocellulose filters which were then soaked in 1.5M NaCl/0.5M NaOH for 2 minutes, 1.5M NaCl/0.5M Tris-Cl pH 8 25 for 5 minutes, 0.2M Tris-Cl pH 7.5/2X SSC for one minute, and baked for 2 hours in an 80°C vacuum oven. Filters were prehybridized in 6X SSC, 5X Denhardt's, 20mM NaH₂PO₄, 500ug/ml salmon sperm DNA for two hours at 42°C. Hybridizations were performed in 0.4% SDS, 6X SSC, 20mM 30 NaH₂PO₄, 500ug/ml salmon sperm DNA for 18 hours at 42°C after the addition ³²P-labelled oligonucleotide. After hybridization, filters were rinsed 3 times with 6X SSC, 0.1% SDS, washed for 10 minutes at room temperature, and washed for 5 minutes at 58°C. Filters were then exposed 35 to X-ray film at -70°C.

Plaques hybridizing with the oligonucleotide probe were cored from plates and resuspended in SM buffer

(100mM NaCl, 8mM MgSO₄, 50mM Tris-Cl pH 7.5, 0.01% gelatin) containing 4% chloroform. Dilutions of such phage stocks were used to infect XL-1 Blue, plaques were transferred to nitrocellulose, and the filters were 5 hybridized with ³²P-labelled oligonucleotides. Well isolated positive plaques were selected and subjected to two additional rounds of purification as just described.

3. Isolation of SERA cDNA-containing Plasmids From Positive Phage Clones.

10 SERA cDNAs in the pBluescript plasmid vector (Stratagene) were obtained by an *in vivo* excision protocol developed for use with the lambda ZAPII vector (Stratagene). Briefly, purified recombinant lambda phage stocks were incubated with XL-1 Blue cells and R408 15 filamentous helper phage for 15 minutes at 37°C. After the addition of 2X YT media (1% NaCl, 1% yeast extract, 1.6% Bacto-tryptone), incubation was continued for 3 hours at 37°C followed by 20 minutes at 70°C. After centrifugation, filamentous phage particles containing 20 pBluescript phagemid (with cDNA insert) were recovered in the supernatant. Dilutions of the recovered filamentous phage stock were mixed with XL-1 Blue and plated to obtain colonies containing pBluescript plasmids with SERA cDNA inserts.

25 4. Generation of Malaria cDNA by PCR.

By use of the polymerase chain reaction (PCR), the 5' portion of the coding sequence of SERA was amplified with specific oligonucleotide primers and first strand cDNA as template (Saiki et al. 1988, Frohman et al. 30 1988). SERA-specific first strand cDNA was synthesized by reverse transcriptase using the reaction conditions described above and specific oligonucleotides as primers. RNA was subsequently eliminated by treatment with RNase A prior to PCR. The GeneAmp DNA amplification kit (Perkin 35 Elmer Cetus, Norwalk, CT.) was used for PCR. Briefly, first strand cDNA in 50mM KCl, 10mM Tris-Cl pH 8.3, 1.5mM MgCl₂, 0.01% gelatin was mixed with 200uM each dNTP, 1uM

of each primer, and 2.5 units Tag polymerase. Reactions were processed in a Thermal Cycler (Perkin Elmer Cetus) with 1 cycle of denaturation, annealing, and extension at 94°C for 2 minutes, 43°C for 3 minutes, and 72°C for 40 minutes; 40 cycles at 94°C for 1 minute, 43°C for 2 minutes, and 72°C for 4 minutes followed by a final extension at 72°C for 20 minutes.

The inclusion of restriction sites in primers used for PCR allowed the cloning of amplified SERA cDNA into 10 plasmid vectors. Clones containing cDNAs derived from two independent PCRs were obtained for each SERA cDNA that was amplified in order to control for Tag polymerase errors.

B. Results.

15 1. Isolation, cloning and characterization of SERA cDNA.

We have isolated overlapping cDNA clones spanning the SERA coding sequence from the FCR3 strain of *P. 20 falciparum*. The p126.6 cDNA, which extends from the EcoRI site at position 1892 (numbering based on SERP I gene of FCBR strain; Knapp et al., 1989) through the 3' end of the coding sequence, was isolated from the blood stage cDNA Lambda ZAPII cDNA library by hybridization to 25 a SERA-specific oligonucleotide JAT2 (SEQ ID NO:115) (5'-GTCTCAGAACGTGTTCATGT-3'), which is derived from the 3' end of the SERA coding sequence (Bzik et al., 1989; Knapp et al., 1989). Clones derived from the 5' end of the SERA coding sequence were obtained by PCR with primers 30 JAT15 (SEQ ID NO:116) (5'-CACGGATCCATGAAGTCATATATTCCTT-3') and JAT16 (SEQ ID NO:117) (5'-GTGAAGCTTAATCCATAATCTTCAATAATT-3') and SERA first strand cDNA template (obtained with oligonucleotide primer JAT17 (SEQ ID NO:118) (5'-GTGAAGCTTTATACATAACAGAAATAACA-3')) 35 and were cloned into pUC19 (New England Biolabs, Beverly, MA.). These 1923 bp cDNAs extend from the initiation codon to a point 31 bp 3' of the internal EcoRI site (position 1892). One such cDNA, p126.8, was found by DNA

sequence analysis to contain a *Taq* polymerase error at nucleotide 1357. This error, an A to G substitution, resides within the 315 bp KpnI/NdeI restriction fragment. A second SERA 5' cDNA, p126.9, has no mutations within 5 this KpnI/NdeI fragment. An unmutated 5' SERA cDNA was generated by replacing the 315 bp KpnI/NdeI fragment in p126.8 with the analogous fragment from p126.9 to generate p126.14. Full length SERA cDNA was generated by ligating the p126.14 5' cDNA as an XmaI/EcoRI fragment 10 into a partial EcoRI/XmaI digested p126.6 vector fragment to generate p126.15.

The complete nucleotide sequence of the p126.15 SERA cDNA insert was determined and is shown in Figures 21A and 21B (SEQ ID NO:119) along with the predicted amino 15 acid sequence (SEQ ID NO:120). This cDNA contains a 2955 bp open reading frame encoding 984 amino acids that is identical to the SERA allele II gene in the FCR3 strain and the FCBR SERP I gene (Li et al., 1989, Knapp et al., 1989).

20 The SERA cDNA was isolated from p126.15 as a 3 Kb XmaI/EcoRV fragment and the XmaI end ligated into an XmaI/BglII digested pCOPCS-5H vector fragment. DNA polymerase I Klenow fragment was used to fill in the pCOPCS-5H BglII site which was subsequently ligated to 25 the EcoRV end to generate p126.16. In this plasmid, SERA is under the control of the early/late vaccinia H6 promotor.

2. Modification of SERA cDNA.

The 3' end of the SERA cDNA was modified to place a 30 vaccinia early transcription termination signal (T₅NT; Yuen and Moss, 1987) and a series of restriction sites (XhoI, SmaI, SacI) immediately after the TAA termination codon. This was accomplished by PCR with oligonucleotides JAT51 (SEQ ID NO:121) (5'- 35 TAGAATCTGCAGGAACCTCAA-3'), JAT52 (SEQ ID NO:122) (5'- CTACACGAGCTCCGGGCTCGAGATAAAATTATACATAACAGAAATAACATTC- 3'), and plasmid p126.16 as template. The resulting ~300

bp amplified fragment was cloned as a PstI/SacI fragment into p126.16 digested with PstI and SacI to generate p126.17.

The 5' end of the SERA cDNA in p126.17 was modified 5 to place several restriction sites (HindIII, SmaI, BamHI) and the 42K entomopox promotor before the ATG initiation codon. This was accomplished by PCR with oligonucleotides JAT53 (SEQ ID NO:123) (5'-
CTAGAGAAGCTTCCCAGGATCCTCAAAATTGAAAATATAATTACAATATAAAATG
10 AAGTCATATATTCCTTGT-3'), JAT54 (SEQ ID NO:124) (5'-
ACTTCCGGGTTGACTTGCT-3'), and plasmid p126.16 as template. The resulting 250 bp amplified fragment was cloned as a HindIII/HindII fragment into p126.17 digested with HindIII and HindII to generate p126.18. This plasmid 15 contains a cassette consisting of the SERA cDNA controlled by the 42K entomopox promotor, with a vaccinia early transcription termination signal, and flanked by restriction sites at the 5' (HindIII, SmaI, BamHI) and 3' (XhoI, SmaI, SacI) ends.

20 The 42K promotor/SERA cassette was isolated from p126.18 as a BamHI/XhoI fragment and cloned into a BamHI/XhoI digested pSD553 vector fragment. The resulting plasmid is designated p126.ATI.

3. Generation of p126.C3.

25 The 42K/SERA expression cassette was isolated from p126.ATI as a BamHI/XhoI fragment and cloned into a BamHI/XhoI-digested VQCP3L vector fragment. The resulting plasmid, designated p126.C3, is an ALVAC C3 donor plasmid.

30 4. Derivation of pSD553.

The pSD553 vaccinia donor plasmid was used for the generation of p126.ATI. It contains the vaccinia K1L host range gene (Gillard et al., 1986) within flanking Copenhagen vaccinia arms, replacing the ATI region (orfs 35 A25L, A26L; Goebel et al., 1990a,b). pSD553 was constructed as follows.

Left and right vaccinia flanking arms were

constructed by PCR using pSD414, a pUC8-based clone of vaccinia SalI B (Goebel et al., 1990a,b) as template. The left arm was synthesized using synthetic deoxyoligonucleotides MPSYN267 (SEQ ID NO:85) (5'-GGGCTGAAGCTTGCTGGCCGCTCATTAGACAAGCGAATGAGGGAC-3') and 5 MPSYN268 (SEQ ID NO:86) (5'-AGA TCT CCC GGG CTC GAG TAA TTA ATT AAT TTT TAT TAC ACC AGA AAA GAC GGC TTG AGA TC-3') as primers. The right arm was synthesized using synthetic deoxyoligonucleotides MPSYN269 (SEQ ID NO:87) 10 (5'-TAA TTA CTC GAG CCC GGG AGA TCT AAT TTA ATT TAA TTT ATA TAA CTC ATT TTT TGA ATA TAC T-3') and MPSYN270 (SEQ ID NO:88) (5'-TAT CTC GAA TTC CCG CGG CTT TAA ATG GAC GGA ACT CTT TTC CCC-3') as primers. The two PCR-derived DNA fragments containing the left and right arms were 15 combined in a further PCR reaction. The resulting product was cut with EcoRI/HindIII and a 0.9kb fragment isolated. The 0.9kb fragment was ligated with pUC8 cut with EcoRI/HindIII, resulting in plasmid pSD541. The polylinker region located at the vaccinia deletion locus 20 was expanded as follows. pSD541 was cut with BglII/XhoI and ligated with annealed complementary synthetic deoxyoligonucleotides MPSYN333 (SEQ ID NO:125) (5'-GAT CTT TTG TTA ACA AAA ACT AAT CAG CTA TCG CGA ATC GAT TCC CGG GGG ATC CGG TAC CC-3')/MPSYN334 (SEQ ID NO:126) (5'-TCG AGG GTA CCG GAT CCC CCG GGA ATC GAT TCG CGA TAG CTG ATT AGT TTT TGT TAA CAA AA-3') generating plasmid pSD552. 25 The K1L host range gene was isolated as a 1kb BglII(partial)/HpaI fragment from plasmid pSD452 (Perkus et al., 1990). pSD552 was cut with BglII/HpaI and 30 ligated with the K1L containing fragment, generating pSD553.

5. Derivation of VQCP3L.

The VQCP3L ALVAC donor plasmid was used for the generation of p126.C3 and was constructed as follows. 35 Insertion plasmid VQCP3L was derived as follows. An 8.5kb canarypox BglII fragment was cloned in the BamHI site of pBS-SK plasmid vector to form pWW5. Nucleotide

sequence analysis of a 7351 bp subgenomic fragment from ALVAC containing the C3 insertion site is presented in Figs. 14A to 14C (SEQ ID NO:127). The C3 ORF is located between nucleotides 1458 to 2897. In order to construct 5 a donor plasmid for insertion of foreign genes into the C3 locus with the complete excision of the C3 open reading frame, PCR primers were used to amplify the 5' and 3' sequences relative to C3. Primers for the 5' sequence were RG277 (SEQ ID NO:128) (5'-
10 CAGTTGGTACCACTGGTATTTATTCAG-3') and RG278 (SEQ ID NO:129) (5'-
TATCTGAATTCCCTGCAGCCCGGGTTTTATAGCTAATTAGTCAAATGTGAGTTAATA
TTAG-3'). Primers for the 3' sequences were RG279 (SEQ ID NO:130)
15 (5' TCGCTGAATTCGATATCAAGCTTATCGATTTTATGACTAGTTAACAAATAAA
AAGCATACAAGC-3') and RG280 (SEQ ID NO:131) (5'-
TTATCGAGCTCTGTAACATCAGTATCTAAC-3'). The primers were designed to include a multiple cloning site flanked by 20 vaccinia transcriptional and translational termination signals. Also included at the 5'-end and 3'-end of the left arm and right arm were appropriate restriction sites (Asp718 and EcoRI for left arm and EcoRI and SacI for right arm) which enabled the two arms to ligate into Asp718/SacI digested pBS-SK plasmid vector. The 25 resultant plasmid was designated as pC3I. A 908 bp fragment of canarypox DNA, immediately upstream of the C3 locus was obtained by digestion of plasmid pWW5 with NsiI and SspI. A 604 bp fragment of canarypox DNA was derived by PCR using plasmid pWW5 as template and 30 oligonucleotides CP16 (SEQ ID NO:132) (5'-
TCCGGTACCGCGGCCGCAGATATTTGTTAGCTCTGC-3') and CP17 (SEQ ID NO:133) (5'-TCGCTCGAGTAGGATAACCTACCTACCTACG-3'). The 604 bp fragment was digested with Asp718 and XhoI (sites present at the 5' ends of oligonucleotides CP16 35 and CP17, respectively) and cloned into Asp718-XhoI digested and alkaline phosphatase treated IBI25 (International Biotechnologies, Inc., New Haven, CT)

generating plasmid SPC3LA. SPC3LA was digested within IBI25 with EcoRV and within canarypox DNA with NsiI, and ligated to the 908 bp NsiI-SspI fragment generating SPCPLAX which contains 1444 bp of canarypox DNA upstream 5 of the C3 locus. A 2178 bp BglII-StyI fragment of canarypox DNA was isolated from plasmids pXX4 (which contains a 6.5 kb NsiI fragment of canarypox DNA cloned into the PstI site of pBS-SK. A 279 bp fragment of canarypox DNA was isolated by PCR using plasmid pXX4 as 10 template and oligonucleotides CP19 (SEQ ID NO:134) (5'-TCGCTCGAGCTTCCTGACAATAACATAG-3') and CP20 (SEQ ID NO:135) (5'-TAGGAGCTTTATACTACTGGGTTACAAC-3'). The 279 bp fragment was digested with XhoI and SacI (sites present at the 5' ends of oligonucleotides CP19 and CP20, 15 respectively) and cloned into SacI-XhoI digested and alkaline phosphatase treated IBI25 generating plasmid SPC3RA. To add additional unique sites to the polylinker, pC3I was digested within the polylinker region with EcoRI and ClaI, treated with alkaline 20 phosphatase and ligated to kinased and annealed oligonucleotides CP12 (SEQ ID NO:136) (5'-AATTCCTCGAGGGATCC-3') and CP13 (SEQ ID NO:137) (5'-CGGGATCCCTCGAGG-3') (containing an EcoRI sticky end, XhoI site, BamHI site and a sticky end compatible with ClaI) 25 generating plasmid SPCP3S. SPCP3S was digested within the canarypox sequences downstream of the C3 locus with StyI and SacI (pBS-SK) and ligated to a 261 bp BglII-SacI fragment from SPC3RA and the 2178 bp BglII-StyI fragment from pXX4 generating plasmid CPRAL containing 2572 bp of 30 canarypox DNA downstream of the C3 locus. SPCP3S was digested within the canarypox sequences upstream of the C3 locus with Asp718 (in pBS-SK) and AccI and ligated to a 1436 bp Asp718-AccI fragment from SPCPLAX generating plasmid CPLAL containing 1457 bp of canarypox DNA 35 upstream of the C3 locus. The derived plasmid was designated as SPCP3L. VQCP3L was derived from pSPCP3L by digestion with XmaI, phosphatase treating the linearized

plasmid, and ligation to annealed, kinased oligonucleotides CP23 (SEQ ID NO:138) (5'-CCGGTTAATTAAATTAGTTATTAGACAAGGTGAAAACGAAACTATTGTAGCTTAATT AATTAGGTCACC-3') and CP24 (SEQ ID NO:139) (5'-CCGGGGTCGACCTAATTAACTAACAAATAGTTCGTTTCACCTGTCTAATA ACTAAATTAAATTAA-3').

DNA sequence analysis of pH6.CEA.C3 revealed a one nucleotide deletion (T) at position 1203 of the CEA coding sequence (eliminating a HindII site) which 10 occurred during a previous cloning step. This deletion was corrected by replacing a 1047 nucleotide MscI fragment (extending from position 501 to 1548) from pH6.CEA.C3 with the analogous, unmutated MscI fragment from pGEM.CEA. The resulting plasmid was designated 15 pH6.CEA.C3.2.

CEA has been inserted into ALVAC by recombination between NotI-linearized pH6.CEA.C3.2 donor plasmid and ALVAC rescuing virus. Recombinants containing CEA have been identified by plaque hybridization with a DNA probe 20 derived from the CEA coding sequence (an NruI/XhoI fragment containing the full length CEA coding sequence).

A NruI/XhoI fragment containing the 3' end of the H6 promotor linked to the full length CEA coding sequence was isolated from pH6.CEA.C3.2. This fragment was 25 ligated to an NruI/XhoI-digested pSPHA.H6 vector fragment, which was derived from the PSD544 HA donor plasmid by the insertion of a fragment containing the H6 promotor. The resulting plasmid was designated pH6.CEA.HA and contains the CEA coding sequence linked to 30 the regenerated H6 promotor. The pH6.CEA.HA donor plasmid directs insertion of the H6/CEA expression cassette to the HA site of NYVAC. Transcription of CEA is oriented from left to right.

Plasmid PSD544 was derived as follows. PSD456 is a 35 subclone of Copenhagen vaccinia DNA containing the HA gene (A56R; Goebel et al., 1990a,b) and surrounding regions. PSD456 was used as template in polymerase chain

reactions for synthesis of left and right vaccinia arms flanking the A56R ORF. The left arm was synthesized using synthetic oligodeoxynucleotides MPSYN279 (SEQ ID NO:140) (5' CCCCCCCGAATTCGTCGACGATTGTTATGATGGCAAGAT 3') and MPSYN280 (SEQ ID NO:141) (5'- CCCGGGGATCCCTCGAGGGTACCAAGCTTAATTAATTAAATTAGTATAAAAAGT GATTTATTTT -3') as primers. The right arm was synthesized using MPSYN281 (SEQ ID NO:142) (5'- AAGCTTGGTACCCCTCGAGGGATCCCCGGGTAGCTAGCTAATTTCTTTACGTAT 10 TATATATGTAATAAACGTTC -3') and MPSYN312 (SEQ ID NO:143) (5'- TTTTTCTGCAGGTAAGTATTTAAACTCTAACACC -3') as primers. Gel-purified PCR fragments for the left and right arms were combined in a further PCR reaction. The resulting product was cut with EcoRI/HindIII. The 15 resulting 0.9 kb fragment was gel-purified and ligated into pUC8 cut with EcoRI/HindIII, resulting in plasmid pSD544. Figures 22 and 23 present the nucleotide sequences of the H6/CEA expression cassettes and flanking regions in plasmids pH6.CEA.C3.2 and pH6.CEA.HA, 20 respectively (SEQ ID NO:144/145, respectively). In Fig. 22, the H6 promoter begins at position 57. The CEA start codon begins at position 181 and the stop codon ends at position 2289. Positions 1 through 58 and 2290 through 2434 flank the H6/CEA expression cassette. In Fig. 23, 25 the H6 promotor begins at position 60. The CEA start codon begins at position 184 and the stop codon ends at position 2292. Positions 1 through 59 and 2293 through 2349 flank the H6/CEA expression cassette.

Example 18 - MURINE IL-2 INTO ALVAC AND NYVAC

30 Insertion of murine IL-2 into ALVAC. Plasmid pmut-1 (ATCC No. 37553) contains the murine IL-2 gene from American Type Culture Collection, Rockville, MD. The IL-2 gene was placed under the control of the vaccinia H6 promoter (Perkus et al., 1989) and the IL-2 3' noncoding 35 end was removed in the following manner.

Template pRW825, containing the H6 promoter and a nonpertinent gene, was used in a polymerase chain

reaction (PCR) with primers MM104 (SEQ ID NO:146)
5'ATCATCGGATCCCTGCAGCCGGGTTAATTAAATTAGTGATAC 3' and MM105
(SEQ ID NO:147) 5'
GAGCTGCATGCTGTACATTACGATAACAACTAACCGGA 3'. The 5' end of
5 MM104 contains BamHI, PstI and SmaI sites followed by a
sequence which primes from the H6 promoter 5' end toward
the 3' end. The 5' end of MM105 overlaps the IL-2 5' end
and MM105 primes from the H6 promoter 3' end toward the
5' end. The resultant 228 base pair PCR derived fragment
10 contains the H6 promoted 5' most base pairs of IL-2.

Template plasmid pmut-1 was used in a second PCR
with primers MM106 (SEQ ID NO:148) 5'
CGTTAAGTTGTATCGTAATGTACAGCATGCAGCTG 3' and MM107 (SEQ ID
NO:149) 5' GAGGAGGAATTCCCCGGGTTATTGAGGGCTTGTGAGA 3'.
15 The 5' end of MM106 overlaps the 3' end of the H6
promoter and primes from the IL-2 5' end toward the 3'
end. The 5' end of MM107 contains EcoRI and SmaI sites
followed by a sequence which primes from the IL-2 3' end
toward the 5' end. The resultant 546 base pair PCR
20 derived fragment was pooled with the above 228 base pair
PCR product and primed with MM104 and MM107. The
resultant 739 base pair PCR derived fragment, containing
the H6 promoted IL-2 gene, was digested with BamHI and
EcoRI, generating a 725 base pair fragment, for insertion
25 between the BamHI and EcoRI sites of pBS-SK (Stratagene,
LaJolla, California), yielding pMM151.

The 755 base pair pMM151 BamHI-XhoI fragment
containing the H6 promoted IL-2 gene was inserted between
the BamHI and XhoI sites of the C3 vector pCP3LSA-2. The
30 resultant plasmid pMM153, contains the H6 promoted IL-2
gene in the C3 locus.

The nucleotide sequence of murine IL-2 from the
translation initiation codon through the stop codon is
given in Figure 24 (SEQ ID NO:150).

35 C3 vector plasmid pCP3LSA-2 was derived in the
following manner. Plasmid SPCP3L (Example 17) was
digested with NsiI and NotI and a 6433 bp fragment

isolated and ligated to annealed oligonucleotides CP34 (SEQ ID NO:151) 5' GGCCGCGTCGACATGCA 3' and CP35 (SEQ ID NO:152) 5' TGTGACGC 3', generating plasmid pCP3LSA-2.

Recombination between donor plasmid pMM153 and ALVAC 5 rescuing virus generated recombinant virus vCP275, which contains the vaccinia H6 promoted murine IL-2 gene in the C3 locus.

Insertion of murine IL-2 into NYVAC. Plasmid pMM151, defined above, was digested with BamHI/XhoI and a 10 755 base pair fragment containing the H6 promoted IL-2 gene was isolated. This BamHI/XhoI fragment was inserted between the BamHI and XhoI sites of the NYVAC TK vector pSD542. The resultant plasmid pMM154, contains the H6 promoted IL-2 gene in the TK locus.

15 Plasmid pSD542 was derived in the following manner. To modify the polylinker region, TK vector plasmid pSD513 (Example 7) was cut with PstI/BamHI and ligated with annealed synthetic oligonucleotides MPSYN288 (SEQ ID NO:153) 5' GGTGACGGATCCT 3' and MPSYN289 (SEQ ID NO:154) 20 5' GATCAGGATCCGTCGACCTGCA 3', resulting in plasmid pSD542.

Recombination between donor plasmid pMM154 and NYVAC rescuing virus generated recombinant virus vP1239, which contains the H6 promoted murine IL-2 gene in the TK 25 locus.

Expression of murine IL-2 in ALVAC and NYVAC based recombinants. ELISA assay. The level of expression of murine IL-2 produced by ALVAC and NYVAC based recombinants vCP275 and vP1239 was quantitated using an 30 ELISA kit from Genzyme Corporation, Cambridge, MA. (InterTest-2XTM Mouse IL-2 ELISA Kit, Genzyme Corporation, Code # 2122-01). Duplicate dishes containing confluent monolayers of mouse L-929 cells (2×10^6 cells/dish) were infected with recombinant virus vCP275 or vP1239 35 expressing murine IL-2 or infected with ALVAC or NYVAC parental virus. Following overnight incubation at 37°C, supernatants were harvested and assayed for expression of

murine IL-2 using the InterTest-2XTM Mouse IL-2 ELISA Kit as specified by the manufacturer (Genzyme Corporation, Cambridge, MA). The InterTest-2XTM Mouse IL-2 ELISA Kit is a solid-phase enzyme-immunoassay employing the 5 multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from duplicate dishes were averaged. The quantity of murine IL-2 secreted is expressed as pg/ml, which is equivalent to pg/10⁶ cells 10 (Table 23).

Table 23

	<u>Recombinant virus</u>	<u>Murine IL-2 secreted</u>
15	vCP275	160 pg/ml
	vP1239	371 pg/ml

Example 19 - HUMAN IL-2 INTO ALVAC AND NYVAC

20 Insertion of Human IL-2 into ALVAC. Plasmid pTCGF- 11 (ATCC No. 39673) contains the human IL-2 gene from American Type Culture Collection, Rockville, MD. The IL- 2 gene was placed under the control of the vaccinia H6 promoter (Perkus et al., 1989), two codons were 25 corrected, and the IL-2 3' noncoding end was removed in the following manner.

Template plasmid pRW825, containing the H6 promoter and a nonpertinent gene, was used in a polymerase chain reaction (PCR) with primers MM104 (SEQ ID NO:146) 30 5' ATCATCGGATCCCTGCAGCCGGGTTAACCTAATTAGTGATAC 3' and MM109 (SEQ ID NO:155) 5' GAGTTGCATCCTGTACATTACGATACAAACTAACCGGA 3'. The 5' end of MM104 contains BamHI, PstI and SmaI sites followed by a sequence which primes from the vaccinia H6 promoter 5' 35 end toward the 3' end. The 5' end of MM109 overlaps the IL-2 5' end, and MM105 primes from the H6 promoter 3' end toward the 5' end. The resultant 230 base pair PCR derived fragment contains the H6 promoted 5' most base pairs of IL-2.

40 Template plasmid pTCGF-11 was used in a PCR with

primers MM108 (SEQ ID NO:156) 5'
CGTTAAGTTGTATCGTAATGTACAGGATGCAACTC 3' and MM112 (SEQ ID
NO:157) 5'
TTGTAGCTGTGTTTCTTGTAGAACCTGAAGTAGGTGCACTGTTGTGACAAGTGC
5 AAGACTTAGTGCAATGCAAGAC 3'. The 5' end of MM108 overlaps
the 3' end of the H6 promoter and primes from the IL-2 5'
end toward the 3' end. MM112 primes from position 100,
in the human IL-2 sequence (Figure 25), toward the 5'
end. The resultant 118 base pair fragment contains the
10 3' most base pairs of the H6 promoter and 5' 100 bp of
the IL-2 gene.

Plasmid pTCGF-11 from American Type Culture
Collection was sequenced, and the sequence was compared
with the published sequence (Clark, et al., 1984). Two
15 mutations resulting in amino acid changes were
discovered. Oligonucleotide primers MM111 (SEQ ID
NO:158) 5'
TTCTACAAAGAAAACACAGCTACAACACTGGAGCATTACTGGATTTACAGATGAT
TTTGAATGGAATTAAATAATTAC 3' and MM112 were used to correct
20 these two base changes in pTCGF-11.

The corrected nucleotide sequence of human IL-2 from
the translation initiation codon through the stop codon
is given in Figure 25 (SEQ ID NO:159).

Except for a silent G to T change in pTCGF-11 at
25 position 114, the sequence in Figure 25 is the same as
the IL-2 sequence described in Clark, et al., 1984. The
T at position 41 in the sequence in Figure 25 is C in
pTCGF-11, and the codon change is from leu to pro. The T
at position 134 in the sequence in Figure 25 is C in
30 pTCGF-11, and the codon change is from leu to ser. The
predicted amino acid sequences of other human, bovine,
murine, ovine, and porcine IL-2 isolates were compared
with the sequence in Clark, et al., 1984; the codons at
positions 41 and 134 are both conserved as leu.

35 Template pTCGF-11 was used in a PCR with primers
MM110 (SEQ ID NO:160) 5'
GAGGAGGAATTCCCCGGGTCAAGTCAGTGTGAGATGA 3' and MM111. The

5' end of MM110 contains EcoRI and SmaI sites followed by a sequence which primes from the IL-2 3' end toward the 5' end. MM111 primes from position 75 toward the IL-2 3' end. The resultant 400 base pair PCR derived fragment 5 was pooled with the above 230 and 118 base pair PCR products and primed with MM104 and MM110. The resultant 680 base pair PCR derived fragment, containing the vaccinia H6 promoted IL-2 gene, was digested with BamHI and EcoRI and inserted between the BamHI and EcoRI sites 10 of pBS-SK (Stratagene, LaJolla, California), yielding pRW956.

Plasmid pRW956 was digested with BamHI/XhoI and a 700 bp fragment containing the H6 promoted IL-2 gene was isolated. This fragment was inserted between the BamHI 15 and XhoI sites of the C3 vector plasmid pCP3LSA-2 (Example 18). The resultant plasmid, pRW958, contains the H6 promoted IL-2 gene in the C3 locus.

Recombination between donor plasmid pRW958 and ALVAC 20 rescuing virus generated recombinant virus vCP277, which contains the H6 promoted human IL-2 gene in the C3 locus.

Insertion of Human IL-2 into NYVAC. Plasmid pRW956, defined above, was digested with BamHI/XhoI and a 700 base pair fragment was isolated. This fragment, containing the vaccinia H6 promoted human IL-2 gene, was 25 inserted between the BamHI and XhoI sites of the NYVAC TK vector plasmid pSD542 (Example 18). The resultant plasmid, pRW957, contains the H6 promoted human IL-2 gene in the TK locus.

Recombination between donor plasmid pRW957 and NYVAC 30 rescuing virus generated recombinant virus vP1241, which contains the H6 promoted human IL-2 gene in the TK locus.

Expression of human IL-2 in ALVAC and NYVAC based recombinants. ELISA assay. The level of expression of human IL-2 produced by ALVAC and NYVAC based recombinants 35 vCP277 and vP1241 was quantitated using a Human Interleukin-2 ELISA kit from Collaborative Biomedical Products, Inc., Becton Dickinson, Bedford, MA. (IL-ISA 2TM

Cat. No. 30020). Duplicate dishes containing confluent monolayers of human HeLa cells (2×10^6 cells/dish) were infected with recombinant virus vCP277 or vP1241 expressing human IL-2 or infected with ALVAC or NYVAC 5 parental virus. Following overnight incubation at 37°C, supernatants were harvested and assayed for expression of human IL-2 using the IL-ISA 2TM Human Interleukin-2 ELISA kit as specified by the manufacturer (Collaborative Biomedical Products, Inc., Becton Dickinson, Bedford, 10 MA). The IL-ISA 2TM Kit is a solid-phase enzyme-immunoassay employing the multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from duplicate dishes were averaged. The IL-ISA 2TM KitIL-2 quantitates human IL-2 in Biological Response 15 Modifiers Program (BRMP) units (Gerrard et al., 1993). The quantity of human IL-2 secreted is expressed as BRMP u/ml, which is equivalent to BRMP u/ 10^6 cells (Table 24).

Table 24

20 Recombinant virus	Human IL-2 secreted
vCP277	850 BRMP U/ml
vP1241	953 BRMP U/ml

25 Example 20 - MURINE IFN γ INTO ALVAC AND NYVAC

Insertion of Murine IFN γ into ALVAC. Plasmid pms10 was obtained from ATCC (#63170). Plasmid pms10 contains cDNA encompassing the entire mouse IFN γ coding sequence 30 with flanking region cloned into the PstI site of pBR322.

Plasmid pMPI3H contains the vaccinia I3L promoter (Perkus et al., 1985; Schmitt and Stunnenberg, 1988) in pUC8. Plasmid pMPI3H is designed for cleavage at a HpaI site within the promoter and at a site in the downstream 35 polylinker region to allow for downstream addition of the 3' end of the I3L promoter linked to a foreign gene.

Linkage of murine IFN γ gene with I3L promoter;

Construction of pMPI3mIF. Murine IFN γ coding sequences with linkage to I3L promoter were synthesized by PCR

using oligonucleotides MPSYN607 (SEQ ID NO:161) 5'
TAATCATGAACGCTACACACTGC 3' and MPSYN608 (SEQ ID NO:162)
5' CCCGGATCCCTGCAGTTATTGGGACAATCTCTT 3' as primers, and
plasmid pms10 as template. The PCR product was cut with
5 BamHI and a 510 bp fragment was isolated and ligated with
vector plasmid pMP13H cut with HpaI/BamHI. Following
sequence verification, the resulting plasmid was
designated pMP13mIF.

10 Insertion of I3L/murine IFN γ cassette into the C3
locus; construction of pMPC3I3mIF. Plasmid pMP13mIF was
cut with HindIII and blunt ended with Klenow fragment of
E. coli polymerase. The DNA was then cut with BamHI and
a 0.6 kb fragment containing the I3L/murine IFN γ cassette
was isolated. This fragment was ligated with vector
15 plasmid pVQC3LSA-3 cut with SmaI/BamHI, resulting in
insertion plasmid pMPC3I3mIF.

20 Nucleotide sequence of the I3L/murine IFN γ
expression cassette is given in Figure 26 (SEQ ID
NO:163). The start codon for the murine IFN γ gene is at
position 101, and the stop codon is at position 596.

25 Plasmid pVQC3LSA-3 was derived in the following
manner. ALVAC C3 locus insertion plasmid VQCP3L (Example
17) was digested with NsiI and NotI and a 6503 bp
fragment isolated and ligated to annealed
oligonucleotides CP34 (SEQ ID NO:151) 5'
30 GGCGCGTCGACATGCA 3' and CP35 (SEQ ID NO:152) 5'
TGTCGACGC 3', generating plasmid VQCP3LSA-3. (Note:
Plasmid VQCP3LSA-3 is identical to plasmids VQCP3LSA-5
and VQCP3LSA, used in subsequent examples; see, e.g.,
Examples 24, 25, 26.)

Recombination between donor plasmid pMPC3I3mIF and
ALVAC rescuing virus generated recombinant virus vCP271,
which contains the I3L promoted murine IFN γ gene in the
C3 locus.

35 Insertion of Murine IFN γ into NYVAC. Insertion of
I3L/murine IFN γ cassette into TK locus; construction of
pMPTKI3mIF. Plasmid pMP13mIF, defined above, was cut

with HindIII and blunt ended with Klenow fragment of *E. coli* polymerase. The DNA was then cut with BamHI and a 0.6 kb fragment containing the I3L/murine IFN γ cassette was isolated. This fragment was ligated with NYVAC TK vector plasmid pSD542 (Example 18) cut with SmaI/BamHI, resulting in insertion plasmid pMPTKI3mIF.

Recombination between donor plasmid pMPTKI3mIF and NYVAC rescuing virus generated recombinant virus vP1237, which contains the I3L promoted murine IFN γ gene in the 10 TK locus.

Expression of murine IFN γ in ALVAC and NYVAC based recombinants. ELISA assay. The level of expression of murine IFN γ produced by ALVAC and NYVAC based recombinants vCP271 and vP1237 was quantitated using an 15 ELISA kit from Genzyme Corporation, Cambridge, MA. (InterTest- γ Kit, Genzyme Corporation, cat # 1557-00). Duplicate dishes containing confluent monolayers of mouse L-929 cells (2×10^6 cells/dish) were infected with 20 recombinant virus vCP271 or vP1237 expressing murine IFN γ or infected with ALVAC or NYVAC parental virus. Following overnight incubation at 37°C, supernatants were harvested and assayed for expression of murine IFN γ using the InterTest- γ Kit as specified by the manufacturer 25 (Genzyme Corporation, Cambridge, MA). The InterTest- γ Kit is a solid-phase enzyme-immunoassay employing the multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from duplicate dishes were averaged. The quantity of murine IFN γ secreted is 30 expressed as nanograms/ml, which is equivalent to ng/ 10^6 cells (Table 25).

Table 25

<u>Recombinant virus</u>	<u>Murine IFNγ secreted</u>
vCP271	972 ng/ml
vP1237	3359 ng/ml

Biological assay. The biological activity of murine IFN γ expressed by ALVAC and NYVAC based recombinants was quantitated using a standardized IFN γ bio-assay (Vogel et al., 1991). This assay quantitates IFN activity by 5 titrating its ability to protect L-929 cells from VSV (vesicular stomatitis virus)-induced cytopathic effect (CPE).

Confluent monolayers of mouse L-929 cells (2×10^6 cells/dish) were mock-infected or infected with NYVAC, 10 vP1237, ALVAC, or vCP271 at an moi of 5. Dishes were inoculated in duplicate. Following the 1 hour adsorption period, 1 ml of fresh medium was added to each dish and they were incubated overnight at 37°C. The supernatants from both dishes were pooled, filtered through a 0.22 μ m 15 filter and tested for IFN activity as detailed below. Two-fold serial dilutions of supernatants were tested, beginning at undiluted for mock infected, NYVAC, and ALVAC infected dishes, or 1:100 and 1:1000 for vCP271 and vP1237 infected dishes.

20 In the IFN- γ bio-assay, 50 μ l medium was added to all wells of a 96-well plate, followed by 50 μ l of a serial dilution of a stock of commercial murine IFN- γ (Genzyme Corporation, MG-IFN, lot # B3649) or culture supernatant as described above. Next, 50 μ l of L-929 25 cells (3×10^4 cells) were added to each well, and plates were placed at 37°C overnight. After 24 hours, cells were infected with 100 μ l VSV (moi of 0.1). Plates were incubated at 37°C overnight. After 24 hours, CPE was assessed. The well which gave the same CPE as VSV in the 30 absence of interferon was defined as having 1 unit/ml interferon. This method coincided well with the interferon standard.

The interferon concentration in the supernatants is determined as the reciprocal of the dilution which gives 35 similar CPE to the standard at 1 unit/ml interferon. For mock-infected, NYVAC and ALVAC infected cells, less than 20 units/ml interferon is produced. For vP1237, 14,000

units/ml IFN- γ is produced, and for vCP271, 3,600 units/ml IFN- γ is produced. vP1237 produced four-fold greater levels of IFN- γ than vCP271, confirming results seen above by the ELISA assay. The lack of protection 5 conferred by supernatants from mock-infected or parental virus-infected cells shows that the protective activity in supernatants from vP1237- and vCP271-infected cells is IFN- γ . This was confirmed by a neutralization assay which showed that antisera to IFN- γ (Genzyme Corporation 10 monoclonal hamster anti-murine IFN- γ , 1222-00, lot #B3847) but not antisera to murine IFN- α/β (Lee Biomolecular Research, INC., San Diego, CA, No. 25301, lot. #89011) or murine IFN- β (Lee Biomolecular Research, Inc., No. 25101, lot. #87065) was capable of neutralizing 15 the protective activity produced by these recombinants.

Example 21 - HUMAN IFN γ INTO ALVAC AND NYVAC

Insertion of Human IFN γ into ALVAC. Plasmid p52 was obtained from ATCC (No. 65949). Plasmid p52 contains cDNA encoding the carboxy terminal 2/3 of the human IFN γ 20 coding sequence with untranslated 3' region cloned into the PstI site of pBR322.

Linkage of human IFN γ gene with I3L promoter;
Construction of pMPI3hIF.

(A) The missing region of the human IFN γ gene was 25 synthesized using long, overlapping PCR primers, MPSYN615 (SEQ ID NO:164) 5'
TAATCATGAAATATACAAGTTATCTTGGCTTTCAAGCTCTGCATCGTTGGGTT
CTCTTGGCTGTTACTGCCAGGACCCATATGTAAAAGAAC 3' and MPSYN616
(SEQ ID NO:165) 5'
30 TTCTTCAAAATGCCTAAGAAAAGAGTTCCATTATCCGCTACATCTGAATGACCTGCA
TTAAAATATTCCTAAGGTTCTGCTTCTTTACATATGGGTCCCTGGC 3' with
no extraneous template. The end of MPSYN615 is designed
for cloning into the HpaI site of pMPI3H (Example 20).
There are 25 bp of overlap between MPSYN615/MPSYN616. A
35 179 bp PCR product was isolated.

(B) The remainder of human IFN γ gene was synthesized using PCR primers MPSYN617 (SEQ ID NO:166)

5' TCTTTCTTAGGCATTTGAAGAATTGGAAAGAGGAGAGTGACAG 3' and
MPSYN618 (SEQ ID NO:167) 5'
CCCGGATCCCTGCAGTTACTGGGATGCTCTTCGA 3' with plasmid p52 as
template. MPSYN617 has 25 bp overlap with missing
5 region; MPSYN618 is designed for cloning into the
downstream BamHI site of pMPI3H. A ca. 350 bp PCR
fragment was isolated.

(A+B) Combination PCR was performed using isolated
fragments from (A) and (B), above, and external primers
10 MPSYN615 and MPSYN618. The PCR product was digested with
BamHI, and a ca. 510 bp fragment was isolated. This
fragment was cloned into pMPI3H cut with HpaI/BamHI.

Following sequence confirmation, the resulting
plasmid was designated pMPI3hIF. pMPI3hIF contains the
15 human IFN γ gene under the control of the I3L promoter.

Insertion of I3L/human IFN γ cassette into C3 locus;
construction of pMPC3I3hIF. Plasmid pMPI3hIF was cut
with HindIII and blunt ended with Klenow fragment of *E.*
coli polymerase. The DNA was then cut with BamHI and a
20 0.6 kb fragment containing the I3L/human IFN γ cassette
was isolated. This fragment was ligated with ALVAC C3
vector plasmid pVQC3LSA-3 (Example 20) cut with
SmaI/BamHI, resulting in ALVAC insertion plasmid
pMPC3I3hIF.

25 Nucleotide sequence of the I3L/human IFN γ
expression cassette is given in Figure 27 (SEQ ID
NO:168). The start codon for the human IFN γ gene is at
position 101, and the stop codon is at position 599.

Recombination between donor plasmid pMPC3I3hIF
30 and ALVAC rescuing virus generated recombinant virus
vCP278, which contains the I3L promoted human IFN γ gene
in the C3 locus.

Insertion of Human IFN γ into NYVAC. Insertion of
I3L/human IFN γ cassette into TK locus; construction of
35 pMPTKI3hIF. Plasmid pMPI3hIF, described above, was cut
with HindIII and blunt ended with Klenow fragment of *E.*
coli polymerase. The DNA was then cut with BamHI and a

0.6 kb fragment containing the I3L/human IFN γ cassette was isolated. This fragment was ligated with NYVAC TK vector plasmid pSD542 (Example 18) cut with SmaI/BamHI, resulting in NYVAC insertion plasmid pMPTKI3hIF.

5 Recombination between donor plasmid pMPTKI3hIF and NYVAC rescuing virus generated recombinant virus vP1244, which contains the vaccinia I3L promoted human IFN γ gene in the TK locus.

10 **Expression of human IFN γ in ALVAC and NYVAC based recombinants**

ELISA assay

The level of expression of human IFN γ produced by ALVAC and NYVAC based recombinants vCP278 and vP1244 was quantitated using a human Interferon γ ELISA kit from 15 Genzyme Corporation, Cambridge, MA. (InterTest- γ TM Kit, Genzyme Corporation, cat # 1556-00). Duplicate dishes containing confluent monolayers of human HeLa cells (2 x 10⁶ cells/dish) were infected with recombinant virus vCP278 or vP1244 expressing human IFN γ or infected with 20 ALVAC or NYVAC parental virus. Following overnight incubation at 37 C, supernatants were harvested and assayed for expression of human IFN γ using the InterTest- γ Kit as specified by the manufacturer (Genzyme Corporation, Cambridge, MA). The InterTest- γ Kit is a 25 solid-phase enzyme-immunoassay employing the multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from duplicate dishes were averaged. The quantity of human IFN γ secreted is 30 expressed as nanograms/ml, which is equivalent to ng/10⁶ cells (Table 26).

Table 26

Recombinant virus

Human IFN γ secreted

35	vCP278	9 ng/ml
	vP1244	15 ng/ml

Example 22 - Murine IL-2 plus IFN γ into ALVAC and NYVAC

Insertion of Murine IFN γ into C6 locus of ALVAC;
addition to ALVAC-murine IL-2 recombinant virus.

Derivation of C6 insertion vector. ALVAC C6 insertion
5 vector pC6L was derived as follows. A 3.0 kb canarypox
HindIII fragment containing the entire C6 ORF was cloned
into the HindIII site of pBS-SK (Stratagene) to form
plasmid pC6HIII3kb. Nucleotide sequence of the canarypox
insert in pC6HIII3kb is presented in Figure 28 (SEQ ID
10 NO:169). In Figure 28, the C6 ORF is located between
nucleotides 377 to 2254.

Extension of canarypox sequence to the right of
pC6HIII3kb was obtained by sequence analysis of
overlapping canarypox clones. In order to construct a
15 donor plasmid for insertion of foreign genes into the C6
locus with the complete excision of the C6 open reading
frame, flanking 5' and 3' arms were synthesized by using
PCR primers and genomic canarypox DNA as template. The
380 bp 5' flanking arm was synthesized using primers C6A1
20 (SEQ ID NO:170) 5'

ATCATCGAGCTCGCGGCCCTATCAAAAGTCTTAATGAGTT 3' and C6B1
(SEQ ID NO:171) 5'
GAATTCCCTCGAGCTGCAGCCGGGTTTTATAGCTAATTAGTCATTTTCGTAAGT
AAGTATTTTATTTAA 3'. The 1155 bp 3' flanking arm was
25 synthesized using primers C6C1 (SEQ ID NO:172) 5'
CCCGGGCTGCAGCTCGAGGAATTCTTTATTGATTAACTAGTCAAATGAGTATATA
TAATTGAAAAAGTAA 3' and C6D1 (SEQ ID NO:173) 5'
GATGATGGTACCTTCATAAAATACAAGTTGATTAACTTAAGTTG 3'. Left
and right flanking arms synthesized above were combined
30 by PCR reaction using primers C6A1 and C6D1, generating a
full length product of 1613bp. This PCR product was cut
near the ends with SacI/KpnI and cloned into pBS-SK cut
with SacI/KpnI, generating C6 insertion plasmid pC6L.
pC6L contains, in the C6 deletion locus, a multicloning
35 region flanked by translational stop codons and T5NT
transcriptional terminators (Yuen and Moss, 1986). The
sequence of pC6L is presented in Figure 29 (SEQ ID

NO:174). In Figure 29, the multicloning region is located between nucleotide 407 and nucleotide 428.

Annealed synthetic oligonucleotides VQC (SEQ ID NO:175) 5'

5 TTAATCAGGATCCTAATTAAATTAGTTATTAGACAAGGTGAAACGAAACTATTTGTA
GCTTAATTAAATTAGCTGCAGCCCCGGG 3' and VQN (SEQ ID NO:176) 5'
10 CCCGGGCTGCAGCTAATTAAATTAGCTACAAATAGTTCTGTTTCACCTTGTCTAAT
AACTAATTAAATTAGGATCCTGATTAA 3' were ligated into pBS-SK
resulting in an intermediate plasmid. Plasmid pMM117
15 contains a SmaI/EcoRI polylinker fragment from this
intermediate plasmid replacing the SmaI/EcoRI polylinker
of pC6L.

Plasmid pMP42GPT contains the *Escherichia coli*
xanthine-guanine phosphoribosyl transferase gene (Ecogpt
15 gene) (Pratt and Subramani, 1983) under the control of an
entomopox promoter (EPV 42kDa). The 31 bp EPV 42kDa
promoter sequence (SEQ ID NO:177) used in pMP42GPT is 5'
CAAAATTGAAAATATAATTACAATATAAA 3'.

Insertion of 42kDa/Ecogpt cassette into C6 locus:
20 Construction of pMP117gpt-B. Plasmid pMP42GPT was cut
with EcoRI and a 0.7kb fragment containing the
42kDa/Ecogpt expression cassette was isolated. This
fragment was inserted into vector plasmid pMM117 cut with
25 EcoRI in both orientations, generating pMP117gpt-A and
pMP117gpt-B.

Insertion of I3L/murine IFN γ cassette into C6 locus:
construction of pMPC6mIFgpt. Plasmid pMP13mIF (Example
20) was cut with HindIII and blunt ended with Klenow
fragment of *E. coli* polymerase. The DNA was then cut
30 with PstI (partial digest) and a 0.6 kb fragment
containing the I3L/murine IFN γ cassette was isolated.
Vector plasmid pMP117gpt-B was cut with SmaI (partial
digest) and full length linear DNA was isolated. This
was cut with PstI and the largest fragment was isolated.
35 Vector and insert fragments were ligated, resulting in
insertion plasmid pMPC6mIFgpt. In addition to the
I3L/murine IFN γ expression cassette, plasmid pMPC6mIFgpt

contains the 42kDa/Ecogpt expression cassette to allow for selection of recombinants through the use of mycophenolic acid (Boyle and Coupar, 1988; Falkner and Moss, 1988).

5 Recombination was accomplished between donor plasmid pMPC6mIFgpt and rescuing virus vCP275 (Example 18). Recombinant virus are plaque purified. The resultant ALVAC based recombinant virus contains the vaccinia I3L promoted murine IFN γ gene, as well as the EPV 42kDa 10 promoted Ecogpt gene, both in the C6 locus and the vaccinia H6 promoted murine IL-2 gene in the C3 locus.

Insertion of Murine IFN γ and Murine IL-2 into NYVAC.

Insertion of I3L/murine IFN γ cassette into TK locus:
construction of pMPTKm2IF. Plasmid pMPI3mIF (Example 20) 15 was cut with HindIII and blunt ended with Klenow fragment of *E. coli* polymerase. The DNA was then cut with BamHI and a 0.6 kb fragment containing the I3L/murine IFN γ cassette was isolated. This fragment was ligated with vector plasmid pMM154 (Example 18) cut with SmaI/BamHI, 20 resulting in insertion plasmid pMPTKm2IF.

Recombination between donor plasmid pMPTKm2IF and NYVAC rescuing virus generated recombinant virus vP1243, which contains the vaccinia I3L promoted murine IFN γ gene and the vaccinia H6 promoted murine IL-2 gene, 25 both in the TK locus.

Expression of murine IL-2 in ALVAC and NYVAC based recombinants: comparison with recombinants coexpressing murine IL-2 and murine IFN γ . ELISA assay. The level of 30 expression of murine IL-2 produced by ALVAC based recombinants vCP275 and vCP288, and NYVAC based recombinants vP1239 and vP1243 was quantitated using a Murine Interleukin-2 ELISA kit from Collaborative Biomedical Products, Inc., Becton Dickinson, Bedford, MA. 35 (Mouse IL-2 ELISA kit Cat. No. 30032). Duplicate dishes containing confluent monolayers of murine L929 cells (2 x 10⁶ cells/dish) were infected with recombinant virus

vCP275 or vP1239 expressing murine IL-2, recombinant virus vCP288 or vP1243 coexpressing murine IL-2 and murine IFN γ , or infected with ALVAC or NYVAC parental virus. Following overnight incubation at 37 C, 5 supernatants were harvested and assayed for expression of murine IL-2 using the mouse Interleukin-2 ELISA kit as specified by the manufacturer (Collaborative Biomedical Products, Inc., Becton Dickinson, Bedford, MA). The mouse Interleukin-2 ELISA kit is a solid-phase enzyme- 10 immunoassay employing the multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from duplicate dishes were averaged. The mouse Interleukin-2 ELISA kit quantitates murine IL-2 in 15 Biological Response Modifiers Program (BRMP) units (Gerrard et al., 1993). The quantity of murine IL-2 secreted is expressed as BRMP u/ml, which is equivalent to BRMP u/ 10^6 cells (Table 27).

20

Table 27

<u>Recombinant virus</u>	<u>Cytokines expressed</u>	<u>Murine IL-2 secreted</u>
vCP275	mlL-2	1838 BRMP u/ml
vCP288	mlL-2 + mlIFN γ	2124 BRMP u/ml
25 vP1239	mlL-2	5030 BRMP u/ml
vP1243	mlL-2 + mlIFN γ	4353 BRMP u/ml

From the results reported in Table 27, it is evident that co-expression of murine IFN γ does not affect the level of 30 murine IL-2 expression by ALVAC or NYVAC-based recombinants. Also, the level of murine IL-2 expression under the conditions of this assay is approximately twice as high for NYVAC based recombinants as it is for ALVAC based recombinants, in agreement with the results 35 presented in Table 23, which were based on a different murine IL-2 ELISA assay (Intertest-2XTM, Genzyme Corporation, Cambridge, MA).

Example 23 - HUMAN IL-2 PLUS IFN γ INTO ALVAC AND NYVAC

Insertion of Human IFN γ into C6 locus of ALVAC;
addition to ALVAC-Human IL-2 recombinant virus.

Insertion of I3L/human IFN γ cassette into C6 locus;

5 construction of pMPC6I3hIF. Plasmid pMPC6I3hIF (Example 21) was cut with HindIII and blunt ended with Klenow fragment of *E. coli* polymerase. The DNA was then cut with BamHI and a 0.6 kb fragment containing the I3L/human IFN γ cassette was isolated. ALVAC C6 vector plasmid 10 pMM117 (Example 22) was cut with BamHI (partial)/SmaI and the largest fragment was isolated. These fragments were ligated, resulting in insertion plasmid pMPC6I3hIF.

Recombination was accomplished between donor plasmid pMPC6I3hIF and rescuing virus vCP277 (Example 15 19). Recombinants are plaque purified. The resultant ALVAC based recombinant virus contains the vaccinia I3L promoted human IFN γ gene in the C6 locus and the vaccinia H6 promoted human IL-2 gene in the C3 locus (vCP277+IFN γ).

20 Insertion of Human IFN γ and IL-2 into NYVAC.

Insertion of H6/human IL-2 cassette into the TK locus;
construction of pMPTK2hIF. Plasmid pMPTK13hIF (Example 21) was cut with PstI and phosphatased with Shrimp alkaline phosphatase. Plasmid pRW858 (Example 19) was 25 cut with PstI and a 700 bp fragment isolated.

Phosphatased vector and insertion fragment were ligated, resulting in plasmid pMPTK2hIF. In pMPTK2hIF, the two human cytokine gene cassettes are both contained within the NYVAC TK insertion site, oriented in a tail-to-tail 30 orientation. The human IFN γ gene is under the control of the vaccinia I3L promoter, and the human IL-2 gene is under the control of the vaccinia H6 promoter.

Recombination was accomplished between donor plasmid pMPTK2hIF and NYVAC rescuing virus. Recombinants 35 are plaque purified. The resultant NYVAC based recombinant virus contains the vaccinia I3L promoted human IFN γ gene and the vaccinia H6 promoted human IL-2

gene, both in the TK locus (NYVAC+IFN γ +IL-2).

Example 24 - MURINE IL-4 INTO ALVAC AND NYVAC

Murine IL-4 into ALVAC. Plasmid p2A-E3, containing the murine IL-4 gene, (m IL-4) was obtained from the 5 American Type Culture Collection (ATCC No. 37561). The murine IL-4 gene was placed under the control of the vaccinia E3L promoter by PCR as described below.

The vaccinia E3L promoter, a strong early promoter, is located immediately upstream from the 10 vaccinia E3L open reading frame (Goebel et al., 1990).

Nucleotide sequence of the E3L/murine IL-4 expression cassette is presented in Figure 30 (SEQ ID NO:178). In Figure 30, the start codon of the murine IL-4 gene is at nucleotide position 68, and the stop codon 15 is at nucleotide position 488.

The mIL-4 gene was amplified by PCR with oligonucleotide primers MIL45 (SEQ ID NO:179) 5' CTCACCCGGGTACCGAATCGAATAAAAAAATGATAAAGTAGGTTCAAGTTTATTGC TGGTTGTGTTAGTTCTCTAAAAATGGGTCTCAACCCCCAG 3' and MIL43 20 (SEQ ID NO:180) 5' TTAGGGATCCAGATCTCGAGATAAAAAC TACGAGTAATCCATTGCATGATGCTC 3' and plasmid p2A-E3 (ATCC) as template. The resulting fragment contained the mIL-4 gene linked to the vaccinia E3L promotor and flanked by XmaI/KpnI/EcoRI and 25 XhoI/BglII/BamHI sites at the 5' and 3' ends, respectively. The amplified E3L/mIL-4 fragment was digested with XmaI/BamHI and ligated to an XmaI/BamHI-digested pVQCP3LSA-5 vector fragment. Plasmid pVQCP3LSA-5 (same as VQCP3LSA-3, Example 20) is an ALVAC C3 locus 30 insertion plasmid. The resulting C3 donor plasmid was designated pC3.MIL4.2. An expression cassette consisting of the *E. coli* gpt gene linked to the entomopox 42kDa promoter was isolated as a SmaI fragment from plasmid pMP42GPT (Example 22), then cloned into a SmaI-digested 35 pC3.MIL4.2 vector fragment. The resulting plasmid was designated pC3MIL4.gpt.

Recombination was accomplished between donor

plasmid pC3MIL4.gpt and ALVAC rescuing virus using the mycophenolic acid selection system (Example 22). Recombinant virus are plaque purified. The resultant recombinant virus contains the murine IL-4 gene under the 5 control of the vaccinia E3L promoter, as well as the Ecogpt gene under the control of the EPV 42kDa promoter, both at the C3 locus of ALVAC.

Murine IL-4 into NYVAC. The mIL-4 gene was amplified by PCR with primers MIL45 (SEQ ID NO:179) and 10 MIL43 (SEQ ID NO:180) and plasmid p2A-E3 (ATCC) as template. The resulting fragment contained the mIL-4 gene linked to the vaccinia E3L promotor and flanked by XmaI/KpnI/EcoRI and XhoI/BglII/BamHI sites at the 5' and 3' ends, respectively. The amplified E3L/mIL-4 fragment 15 was digested with XmaI/BamHI. NYVAC TK insertion plasmid pSD542 (Example 18) was digested with XmaI/BamHI and ligated to the XmaI/BamHI-digested PCR fragment. The resulting TK donor plasmid was designated pTK-mIL4.

Recombination between donor plasmid pTK-mIL4 20 and NYVAC rescuing virus generated recombinant virus vP1248 which contains the vaccinia E3L promoted murine IL-4 gene in the TK locus.

Example 25 - HUMAN IL-4 INTO ALVAC AND NYVAC

Human IL-4 into ALVAC. Plasmid pcD-hIL-4, 25 containing the human IL-4 gene, was obtained from the American Type Culture Collection (ATCC No. 57593).

PCR fragment PCRhIL4-I was synthesized using plasmid pcD-hIL-4 as template DNA and synthetic oligonucleotides E3LIL4-C (SEQ ID NO:181) 5' 30 GCTGGTTGTGTTAGTTCTCTCTAAAAATGGGTCTCACCTCCAACTG 3' and E3LIL4-D (SEQ ID NO:182) 5' ATCATCTCTAGAATAAAAATCAGCTCGAACACTTGAATATTCCTCTCATG 3' as primers.

Oligonucleotides E3LIL4-A (SEQ ID NO:183) 5' 35 ATCATCAAGCTTGAATAAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTG TTAGTTCTCTAAAA 3' and E3LIL4-B (SEQ ID NO:184) 5' TTTTAGAGAGAACTAACACAAACAGCAATAAAACTGAACCTACTTTATCATTTTTT

ATTC 3' were annealed to generate Fragment II containing the vaccinia E3L promoter sequence (Example 24).

A second fusion PCR product (PCR_hIL4-II) was obtained using PCR fragment PCR_hIL4-I and Fragment II (annealed oligos) as DNA template and E3LIL4-D and E3LIL4-E (SEQ ID NO:185)

5' ATCATCAAGCTTGAATAAAAAATGATAAAGTAGGTCAG 3' as oligonucleotide primers. A complete HindIII/XbaI digest of PCR_hIL4-II yielded an 536 bp fragment which was 10 subsequently isolated. A complete HindIII/XbaI digest of pBS-SK+ (Stratagene) was performed and the 2.9 kb fragment isolated. The isolated fragments were ligated, resulting in plasmid pBShIL4.

15 Figure 31 (SEQ ID NO:186) presents the nucleotide sequence of the expression cassette consisting of the E3L promoted human IL-4 gene. The start codon for the human IL-4 gene is at nucleotide position 62, and the stop codon is at nucleotide position 521.

20 A complete XbaI digest of plasmid pBShIL4 was performed. Ends were filled in using Klenow fragment of *E. coli* polymerase. This linearized plasmid was then digested with XhoI and the 536 bp fragment, containing the E3L promoter and human IL4 gene, was isolated. C3 insertion vector plasmid VQCP3LSA (same as pVQCP3LSA-3, 25 Example 20) was completely digested with XhoI/SmaI and the 6.5 kb fragment isolated. The isolated fragments were ligated, resulting in insertion plasmid pC3hIL4.

30 Recombination between donor plasmid pC3hIL4 and ALVAC rescuing virus generated recombinant virus vCP290, which contains the vaccinia E3L promoted human IL-4 gene in the C3 locus.

35 Human IL-4 into NYVAC. Plasmid pBShIL4 (discussed above) contains the E3L/human IL-4 expression cassette in pBS-SK. A complete XbaI digest of plasmid pBShIL4 was performed. Ends were filled in using Klenow fragment of *E. coli* polymerase. This linearized plasmid was then digested with XhoI and the 536 bp fragment, containing

the E3L promoter and human IL-4 gene, was isolated. NYVAC TK insertion vector plasmid pSD542 (Example 18) was completely digested with XbaI/SmaI and the 3.9 kb fragment isolated. The isolated fragments were ligated, 5 resulting in insertion plasmid pTKhIL4.

Recombination between donor plasmid pTKhIL4 and NYVAC rescuing virus generated recombinant virus vP1250, which contains the vaccinia E3L promoted human IL-4 gene in the TK locus.

10 Example 26 - HUMAN GMCSF IN ALVAC AND NYVAC

Human GMCSF into ALVAC. Plasmid GMCSF, containing the gene encoding the human granulocyte-macrophage colony-stimulating factor (hGMCSF), was obtained from the American Type Culture Collection (ATCC No. 39754).

15 PCR fragment GMCSF-I was synthesized using plasmid GMCSF as template DNA and E3LGMC-A (SEQ ID NO:187)
5' GCTGGTTGTGTTAGTTCTCTCTAAAAATGTGGCTGCAGAGCCTGCTG 3' and E3LGMC-B (SEQ ID NO:188)
5' ATCATCCTCGAGATAAAAATCACTCCTGGACTGGCTCCCAGCAGTCAAAGGGG
20 3' as oligonucleotide primers.

Synthetic oligonucleotides E3LSMA-B (SEQ ID NO:189)
5'
ATCATCCCCGGGAATAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTG
TTAGTTCTCTCTAAAAA 3' and E3LIL4-B (SEQ ID NO:184; Example
25 25) were annealed to generate fragment GMCSF-P containing the vaccinia E3L promoter sequence.

A fusion PCR product (GMCSF-II) was obtained using fragments GMCSF-I and GMCSF-P as DNA templates and E3LSMA-A (SEQ ID NO:190) 5'
30 ATCATCCCCGGGAATAAAAATGATAAAGTAGGTTCAG3' and E3LGMC-B as oligonucleotide primers. A complete XbaI/SmaI digest of GMCSF-II yielded a 0.5 kb fragment which was subsequently isolated. A complete XbaI/SmaI digest of pBS-SK+ was performed and the 2.9 kb fragment isolated.
35 The isolated fragments were ligated, resulting in plasmid pBSGMCSF, which contains the vaccinia E3L/hGMCSF expression cassette.

Nucleotide sequence of the vaccinia E3L/hGMCSF expression cassette is given in Figure 32 (SEQ ID NO:191). In Figure 32, the start codon for hGMCSF is at nucleotide position 62, the stop codon is at nucleotide 5 position 494.

A complete XhoI/SmaI digest of pBSGMCSF (above) was performed and the 0.5 kb fragment, containing the vaccinia E3L promoter and hGMCSF gene, was isolated. ALVAC C3 insertion plasmid VQCP3LSA (Example 20) was 10 completely digested with XhoI/SmaI and the 6.5 kb fragment isolated. The isolated fragments were ligated, resulting in plasmid pC3hGMCSF.

Recombination between donor plasmid pC3hGMCSF and ALVAC rescuing virus generated recombinant virus vCP285, 15 which contains the vaccinia E3L promoted human GMCSF gene in the C3 locus.

Human GMCSF into NYVAC. A complete XhoI/SmaI digest of pBSGMCSF was performed and the 0.5 kb fragment, containing the vaccinia E3L promoter and hGMCSF gene, was 20 isolated. pSD542 (Example 18) was completely digested with XhoI/SmaI and the 3.9 kb fragment isolated. The isolated fragments were ligated, resulting in plasmid pTKhGMCSF.

Recombination between donor plasmid pTKhGMCSF and 25 NYVAC rescuing virus generated recombinant virus vP1246, which contains the vaccinia E3L promoted human GMCSF gene in the TK locus.

Expression of human GMCSF in ALVAC and NYVAC based recombinants. ELISA assay. The level of expression of 30 human GMCSF produced by ALVAC and NYVAC based recombinants vCP285 and vP1246 was quantitated using an ELISA kit from Genzyme Corporation, Cambridge, MA. (Factor-Test Human GM-CSF ELISA Kit, Genzyme Corporation, product code GM-TE.) Duplicate dishes containing 35 confluent monolayers of human HeLa cells (2×10^6 cells/dish) were infected with recombinant virus vCP285 or vP1246 expressing human GMCSF or infected with ALVAC

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or NYVAC parental virus. Following overnight incubation at 37°C, supernatants were harvested and assayed for expression of human GMCSF using the Factor-Test Human GM-CSF ELISA kit as specified by the manufacturer (Genzyme Corporation, Cambridge, MA). The Factor-Test Human GM-CSF ELISA Kit is a solid-phase enzyme-immunoassay employing the multiple antibody sandwich principle. ELISA plates were read at 490 nm. Background from ALVAC or NYVAC samples was subtracted, and values from 10 duplicate dishes were averaged. The quantity of human GMCSF secreted is expressed as picograms(pg)/ml, which is equivalent to pg/10⁶ cells (Table 28).

Table 28

15	<u>Recombinant virus</u>	<u>Human GMCSF secreted</u>
	vCP285	2413 pg/ml
	vP1246	4216 pg/ml
20 <u>Example 27 - HUMAN IL-12 IN ALVAC AND NYVAC</u>		
Derivation of DNA encoding the two subunits of the human IL-12 gene. First strand cDNA synthesis was performed on total RNA isolated from human EBV		
25	transformed cell line GJBCL stimulated 24 hrs. with 100nM Phorbol 12,13-Dibutyrate. Oligonucleotide primers used for PCR amplification of the genes encoding the p35 and p40 subunits (below) were based on the published human IL-12 sequence (Gubler et al., 1991).	
30	The p40 subunit of the human IL-12 gene (hIL12p40) was obtained as PCR fragment PCR J60 using first strand cDNA from cell line GJBCL as template and oligonucleotides JP202 (SEQ ID NO:192) 5'	
	CATCATATCGATGGTACCTCAAAATTGAAAATATATAATTACAATATAAAATGTGTC	
35	ACCAGCAGTTGG 3' and JP189 (SEQ ID NO:193) 5'	
	TACTACGAGCTCTCAGATAGAAATTATCTTTTGGG 3' as primers.	
	PCR J60 was cut with <u>SacI/ClaI</u> and a 1.0 kb fragment was isolated and ligated with pBSSK ⁺ (Stratagene), cut with <u>SacI/ClaI</u> , generating plasmid PBSHIL12p40II. In plasmid	
40	PBSHIL12p40II, hIL12p40 is under the control of the	

entomopox 42kDa promoter (Example 22).

The sequence of the EPV 42kDa/human IL-12 P40 expression cassette is presented in Figure 33 (SEQ ID NO:194). In Figure 33, the initiation codon for the 5 human IL-12 P40 subunit is at nucleotide position 32, the stop codon is at nucleotide position 1017.

The p35 subunit of the human IL-12 gene (hIL12p35) was obtained as PCR fragment PCR J59 using first strand cDNA from cell line GJBC1 as template as oligonucleotides 10 JP186 (SEQ ID NO:195) 5'

CATCATGGTACCTCAAAATTGAAAATATATAATTACAATATAAAATGTGTCCAGCGC GCAGCC 3' and JP201 (SEQ ID NO:196) 5'

15 TACTACATCGATTTAGGAAGCATTCAAGATAG 3' as primers. PCR J59 was cut with Asp718/ClaI and a 0.7 kb fragment was isolated and ligated with pBSSK⁺ (Stratagene), generating plasmid PG2. The hIL12p35 gene was put under the control of the vaccinia E3L promoter (Example 24) by a PCR reaction using plasmid PG2 as template and oligonucleotides JP218 (SEQ ID NO:197) 5'

20 CATCATGGTACCGAATAAAAAATGATAAAAGTAGGTTCAAGTTTATTGCTGGTTGTG TTAGTTCTCTCTAAAAATGTGTCCAGCGCGCAGCC 3' and JP220 (SEQ ID NO:198) 5' CATCATATCGATTTAGGAAGCATTCAAGATAGCTCGTCAC 3' as primers. PCR J62 was cut with Asp718/ClaI and a 0.7 kb fragment was isolated and ligated with pBSSK⁺ 25 (Stratagene), generating plasmid PBSHIL12p35II.

The sequence of the vaccinia E3L/human IL-12 P35 expression cassette is presented in Figure 34 (SEQ ID NO:199). In Figure 34, the initiation codon for the 30 human IL-12 P35 subunit is at nucleotide position 62, the stop codon is at nucleotide position 719.

A cassette containing poxvirus-promoted genes for both subunits of human IL-12 was assembled in pBSSK⁺ by ligating a 0.7kb Asp718/ClaI fragment from PBSHIL12p35II and a 1.0kb Asp718/SacI fragment from PBSHIL12p40II into 35 pBSSK⁺ cut with SacI/ClaI. The resulting plasmid was designated PBSHIL12. In PBSHIL12 the EPV 42kDa/hIL12p40 cassette and the vaccinia E3L/hIL12p35 cassette are

oriented in a head-to-head orientation relative to each other.

Human IL-12 into ALVAC. The combination cassette containing poxvirus-promoted genes for both subunits of 5 human IL-12 was excised as a 1.7kb SacI/ClaI fragment from plasmid PBSHIL12. The fragment was blunt-ended by treatment with the Klenow fragment of *E. coli* polymerase, and cloned into ALVAC C6 vector plasmid pC6L (Example 22) 10 cut with SmaI. The resulting plasmid was designated pC6HIL12.

Recombination was performed between donor plasmid pC6HIL12 and ALVAC rescuing virus. Recombinant virus are plaque purified. The resultant recombinant virus (ALVAC 15 + IL-12) contains both of the human IL-12 genes in the C6 locus of ALVAC.

Human IL-12 into NYVAC. The combination cassette containing poxvirus-promoted genes for both subunits of human IL-12 was excised as a 1.7kb SacI/ClaI fragment from plasmid PBSHIL12. The fragment was blunt-ended by 20 treatment with the Klenow fragment of *E. coli* polymerase, and cloned into NYVAC TK vector plasmid pSD542 (Example 18) cut with SmaI. The resulting plasmid was designated pTKHIL12.

Recombination was performed between donor plasmid 25 pTKHIL12 and NYVAC rescuing virus. Recombinant virus are plaque purified. The resultant recombinant virus (NYVAC + IL-12) contains both of the human IL-12 genes in the TK locus of NYVAC.

Example 28 - MURINE B7 IN ALVAC AND NYVAC

30 Murine B7 into ALVAC. Preparation of cDNA for murine B7. Macrophages from a naive Balb/c mouse spleen were stimulated in vitro with Concanavalin A and LPS. Total RNA from these cells was used as a template for first-strand cDNA synthesis by reverse transcription 35 using oligo dT as a primer. An aliquot of first strand cDNA preparation was used for the specific murine B7 cDNA amplification by PCR using the primers LF32 (SEQ ID

NO:200) 5'

TATCTGGAATTCTATCGCGATATCCGTTAAGTTGTATCGTAATGGCTTGCAATTGT
CAG 3' and LF33 (SEQ ID NO:201) 5'

ATCGTAAAGCTTACTAAAGGAAGACGGTCTG 3'. The specific primers
5 LF32 and LF33 were derived from the published sequence of
murine B7 (Freeman and al., 1991). Nucleotides 5' to the
ATG in LF32 correspond to part of the vaccinia H6
promoter (Perkus et al., 1989). The amplified 951
nucleotide cDNA fragment containing the murine B7 gene
10 was digested by EcoRI and HindIII and subsequently cloned
into the corresponding sites of the plasmid pBSSK⁺
(Statagene). The resulting plasmid, pLF1, was digested
with NruI and XhoI, and a 949 bp fragment containing part
of the vaccinia H6 promoter and the entire murine B7 gene
15 was isolated.

Plasmid pMPC616E6 contains a non relevant gene
under the control of the vaccinia H6 promoter in the
ALVAC C6 insertion locus. Plasmid pMPC616E6 was digested
with NruI and XhoI, and the 4,403 bp NruI-XhoI fragment
20 containing the bulk of the H6 promoter in the ALVAC C6
insertion locus was isolated. This vector fragment was
ligated with the NruI/XhoI fragment from pLF1. The
resulting plasmid was named pLF4.

Nucleotide sequence of the murine B7 gene is
25 given in Figure 35 (SEQ ID NO:202). In Figure 35, the
start codon for the murine B7 gene is at nucleotide 1 and
the stop codon is at nucleotide 919.

Recombination between donor plasmid pLF4 and
ALVAC rescuing virus generated recombinant virus vCP268,
30 which contains the vaccinia H6 promoted murine B7 gene in
the C6 locus.

Murine B7 into NYVAC. Plasmid pSIV12 contains
a nonrelevant gene under the control of the vaccinia H6
promoter in the NYVAC I4L insertion locus. Plasmid
35 pSIV12 was digested with NruI and XhoI, and the 3,557 bp
NruI-XhoI fragment containing the bulk of the H6 promoter
in the NYVAC I4L insertion locus was isolated. This

fragment was ligated to annealed synthetic oligonucleotides LF57 (SEQ ID NO:203) 5' CGACATTTGGATTTCAAGCTTCTACG 3' and LF58 (SEQ ID NO:204) 5' GATCCGTAGAAGCTTGAAATCCAAATGTGCG 3' which contain an 5 internal HindIII site. The resulting plasmid, pLF2, was digested with NruI and HindIII, and a 3,659bp vector fragment was isolated. Plasmid pLF1 (above) was digested with NruI and HindIII, and a 951 bp NruI-HindIII fragment containing part of the vaccinia H6 promoter and the 10 entire murine B7 gene was isolated. These two fragments were ligated, generating plasmid pLF3.

Plasmid pLF3 corresponds to an I4L NYVAC donor plasmid containing the entire murine B7 coding sequence under the control of the vaccinia H6 promoter.

15 Recombination between donor plasmid pLF3 and NYVAC rescuing virus generated recombinant virus vP1230, which contains the vaccinia H6 promoted murine B7 gene in the I4L locus.

20 Surface expression of B7 on murine tumor cells
infected with ALVAC and NYVAC-based recombinants
expressing murine B7. K1735 mouse melanoma cells and CC-
36 mouse colon carcinoma cells were infected with 10 pfu
per cell of NYVAC-B7 (vP1230), ALVAC-B7 (vCP268), or
NYVAC or ALVAC parental virus for 1 hour, washed free of
25 unadsorbed virus by centrifugation, and incubated at
37°C. B16 mouse melanoma cells were treated similarly
except that the cells were infected with 5 pfu of virus
per cell. After a 1 hr (K1735) or overnight (CC-36;B16)
incubation, the cells were washed in PBS by
30 centrifugation and resuspended in 1.0 ml of PBS. To each
cell preparation, 0.005 ml of 1:5 diluted Fc Block
(Pharmingen, San Diego, CA, cat. 01241A; purified anti-
mouse Fcγ II receptor) and 0.1 ml of 1:100 diluted FITC-
rat anti-mouse B7 monoclonal antibody (Pharmingen, cat.
35 01944D) was added. The cells were incubated for 30
minutes at 4°C, washed twice in cold PBS by
centrifugation and analyzed for cell-associated FITC

fluorescence by flow cytometry (Becton-Dickinson FACScan).

Although K1735 cells infected with NYVAC-B7 (vP1230) or ALVAC-B7 (vCP268) for 1 hour showed only 5 slightly higher fluorescence than control uninfected or NYVAC or ALVAC infected cells, B7 expression in recombinant infected CC-36 and B16 cells was remarkable (Figure 36). As demonstrated by the uninfected control cells, none of the three cell lines endogenously 10 expresses murine B7. Clearly, infection of established murine tumor cell lines with NYVAC-B7 (vP1230) or ALVAC-B7 (vCP268), but not the vectors NYVAC or ALVAC, results in high levels of expression of the murine T-lymphocyte co-activator molecule, BB-1/B7.

15 Example 29 - HUMAN B7 IN NYVAC

Preparation of cDNA for human B7.

Macrophages from human peripheral blood were stimulated in vitro with Concanavalin A and LPS. Total RNA from these cells was used as a template for first- 20 strand cDNA synthesis by reverse transcription using oligo dT as a primer. An aliquot of first strand DNA preparation was used for specific human B7 cDNA amplification by PCR using the primers LF62 (SEQ ID NO:205) 5' ATCGTAAGCTTATTATACAGGGCGTACACTTC 3' and LF61bis (SEQ ID NO:206) 5' 25 TATCTGGAATTCTATCGCGATATCCGTTAAGTTGTATCGTAATGGGCCACACACGG AGG 3'.

The specific primers LF62 and LF61bis were derived from the published sequence of human B7 (Freeman and al., 30 1989). Nucleotides 5' to the ATG in LF61bis correspond to part of the vaccinia H6 promoter (Perkus et al., 1989). The amplified 997 nucleotide cDNA fragment containing the murine B7 gene was digested by EcoRI and HindIII and subsequently cloned into the corresponding 35 sites of the plasmid pBSSK⁺ (Stratagene). This plasmid was designated pLF6.

The sequence for the human B7 gene is presented in

Figure 37 (SEQ ID NO:207). In Figure 37, the start codon for the human B7 gene is at nucleotide position 1 and the stop codon is at nucleotide position 865.

Insertion of Human B7 into NYVAC.

5 Plasmid pLF3 (Example 28) was digested with NruI and HindIII and a 3652 bp vector fragment containing the bulk of the H6 promoter in the NYVAC I4L insertion locus was isolated. Plasmid pLF6 (above) was digested with NruI and HindIII, and a 897 bp fragment containing part of the 10 vaccinia H6 promoter and the entire human B7 gene was isolated. These two fragments were ligated, generating plasmid pLF7.

Plasmid pLF7 corresponds to an I4L NYVAC donor 15 plasmid containing the entire human B7 coding sequence under the control of the vaccinia H6 promoter.

Recombination between donor plasmid pLF7 and NYVAC rescuing virus generated recombinant virus vP1245, which contains the vaccinia H6 promoted human B7 gene in the I4L locus.

20 Expression of human B7. FACScan.

Human HeLa cells were infected with recombinant virus vP1245 expressing human B7 or with NYVAC parental virus. A monoclonal antibody specific for human B7 (Anti-BB1(B7), Cat. No. 550024, Becton Dickinson Advanced 25 Cellular Biology, San Jose, CA), was used to detect expression of human B7 on the surface of infected cells by flow cytometry (Becton-Dickinson FACScan) as described in Example 28. B7 was detected on the surface of cells infected with recombinant virus vP1245. B7 was not 30 detected on the surface of uninfected cells or cells infected with NYVAC parental virus.

Immunoprecipitation. NYVAC based recombinant virus vP1245 was assayed for expression of the human B7 gene using immunoprecipitation. Recombinant or parental virus 35 were inoculated onto preformed monolayers of tissue culture cells in the presence of radiolabelled ³⁵S-methionine and treated as previously described (Taylor et

al., 1990). Immunoprecipitation reactions were performed using a monoclonal antibody specific for human B7 (Anti-BB1(B7), Cat. No. 550024, Becton Dickinson Advanced Cellular Biology, San Jose, CA). A protein of 5 between approximately 44 and 54 kDa was precipitated from cells infected with recombinant virus vP1245, in agreement with Freeman et al. (1989). The protein was not immunoprecipitated from uninfected cells or cells infected with NYVAC parental virus.

10 **Example 30 - CO-INSERTION OF MURINE IFN γ AND MURINE B7 INTO ALVAC AND NYVAC**

Co-Insertion of Murine IFN γ and Murine B7 into ALVAC. Recombination was accomplished between donor 15 plasmid pLF4 (Example 28) and rescuing virus vCP271 (Example 20). Recombinant virus are plaque purified. The resultant ALVAC based recombinant virus (ALVAC + IFN γ + B7) contains the vaccinia I3L promoted murine IFN γ gene in the C3 locus and the vaccinia H6 promoted murine B7 20 gene in the C6 locus.

Co-Insertion of Murine IFN γ and Murine B7 into NYVAC. Recombination was accomplished between donor plasmid pMPTKmIF (Example 20) and rescuing virus vP1230 (Example 28). Recombinant virus are plaque purified. 25 The resultant NYVAC based recombinant virus (NYVAC+IFN γ +B7) contains the vaccinia I3L promoted murine IFN γ gene in the TK locus and the vaccinia H6 promoted murine B7 gene in the I4L locus.

30 **Example 31 - INSERTION OF WILDTYPE AND MUTANT FORMS OF MURINE P53 INTO ALVAC**

The gene for the nuclear phosphoprotein p53 is the gene most frequently found to be mutated in a wide variety of human tumors (reviewed in Hollstein et al., 35 1991). NYVAC and ALVAC-based p53 recombinant virus are described in Example 15.

Insertion of wildtype Murine p53 into ALVAC. Plasmid p11-4 containing murine wild-type p53 was received from Arnold Levine (Princeton University,

Princeton, New Jersey). The p53 sequence is described in Pennica et al., (1984). The murine wild-type p53 gene was placed under the control of the vaccinia H6 promoter and the p53 3' non coding end was removed with PCR-derived fragments.

A fragment containing the H6 promoted 5' end of the p53 gene fused to the 3' end of the p53 gene was generated by several PCRs as described below.

PCR I: Plasmid pRW825, containing the H6 promoter and a nonpertinent gene, was used as template with oligonucleotides MM080 (SEQ ID NO:208) 5'
ATTATTATTGGATCCTTAATTAATTAGTGATACGC 3' and MM081 (SEQ ID NO:209) 5'
CTCCTCCATGGCAGTCATTACGATACAAACTAAC 3' producing a 228bp fragment containing the H6 promoter and the 5'-most base pairs of the murine p53 gene. MM080 anneals to the 5' end of the H6 promoter and primes toward the 3' end. MM081 anneals to the 3' end of the H6 promoter and primes toward the 5' end.

PCR II: Plasmid p11-4 was used as template with oligonucleotides MM082 (SEQ ID NO:210) 5'
CGTTAAGTTGTATCGTAATGACTGCCATGGAGGAGTC 3' and MM083 (SEQ ID NO:211) 5'
TAGTAGTAGTAGCTTCTGGAGGAAGTAGTTCC 3' to generate a 129bp fragment with the 3'-end of the H6 promoter, the 5' end of the p53 gene followed by 15bp which overlaps PCR fragment PCRIII (described below). MM082 contains the 3' end of the H6 promoter and primes from the 5' end of the murine p53 gene. MM083 anneals to position 97 (Figure 38) of the murine p53 gene and primes toward the 5' end.

PCRIII: Plasmid p11-4 was used as template with oligonucleotides MM084 (SEQ ID NO:212) 5'
CAGAAGCTACTACTACTACCCACCTGCACAAGCGCC 3' and MM085 (SEQ ID NO:213) 5'
35 AACTACTGTCCCGGGATAAAAATCAGTCTGAGTCAGGCCAC 3' to generate a 301bp fragment. The 301bp PCR-derived fragment contains the 3' end of the p53 gene, and the 5'

end overlaps the 3' end of the PCRII product. MM084 (SEQ ID NO:212) primes from position 916 of the murine p53 gene toward the 3' end. MM085 (SEQ ID NO:213) primes from position 1173 toward the p53 gene 5' end. The three 5 PCR products were pooled and primed with MM080 and MM085. The resultant 588bp fragment contains a BamHI site followed by the H6 promoted 5' end of the p53 gene fused to the p53 gene 3' end followed by a SmaI site; the 5' end of the p53 gene ends at the XhoI site at position 37, 10 and the 3' end starts at the SacII site at position 990 (Figure 38). The 588bp PCR-derived fragment was digested with BamHI and SmaI generating a 565bp fragment which was inserted into BamHI/SmaI digested pNC5LSP5 (described below). The resultant plasmid, designated pMM136, was 15 digested with KspI and XhoI to remove a 149bp fragment, and the 953bp KspI/XhoI fragment from p11-4 was inserted. The resultant plasmid, pMM148, contains the H6 promoted wild-type murine p53 in the ALVAC C5 insertion locus.

The construction of pNC5LSP5 is as follows. A C5 20 insertion vector plasmid pC5LSP (Example 14) was digested with EcoRI, treated with alkaline phosphatase and ligated to self-annealed oligonucleotide CP29 (SEQ ID NO:102) 5' AATTGCGGCCGC 3', then digested with NotI and linear purified followed by self-ligation. This procedure 25 introduced a NotI site to pC5LSP, generating pNC5LSP5.

The nucleotide sequence of the wildtype murine p53 gene is presented in Figure 38 (SEQ ID NO:214). The start codon is at position 1 and the stop codon is at position 1171.

30 Recombination between donor plasmid pMM148 and ALVAC rescuing virus generated recombinant virus vCP263. vCP263 contains the wild type murine p53 gene under the control of the vaccinia H6 promoter in the C5 locus.

Insertion of a mutant form of Murine p53 into ALVAC.
35 Plasmid pSVK215 containing a mutant form of the murine p53 gene was received from Arnold Levine (Princeton University, Princeton, New Jersey). The mutation in

pSVKH215 changes the sequence GTAC of the murine p53 coding sequence (Figure 38) nt positions 643 through 646 to CCAAGCTTGG. The insertion between nt positions 643 and 646 changes the predicted amino acid coding sequence 5 from val-pro to pro-ser-leu-ala; and the insertion replaces a KpnI site with a HindIII site. The construction of pSVKH215 is described in Tan et al., (1986).

Plasmid pMM136 (described above) contains the 10 vaccinia H6 promoted 5' end of the p53 gene fused to the 3' end of the p53 gene in an ALVAC C5 locus insertion plasmid. pMM136 was digested with KspI and XhoI to remove 149bp, and the 960bp KspI/XhoI fragment containing the mutation described above from pSVKH215 was inserted. 15 The resultant plasmid, pMM149, contains the H6 promoted murine mutant p53 gene in the C5 locus.

Recombination between donor plasmid pMM149 and ALVAC rescuing virus generated recombinant virus vCP267. vCP267 contains the mutant form of the murine p53 gene 20 under the control of the vaccinia H6 promoter in the C5 locus.

Example 32 - INSERTION OF MUTANT FORMS OF HUMAN P53 INTO ALVAC AND NYVAC

25 Mutant forms of Human p53 into ALVAC.

Figure 18 (Example 15) presented the sequence of the vaccinia H6 promoted human wild type p53 gene cassette in an ALVAC-based recombinant, vCP207. In this example, to facilitate description of the mutant forms of the human 30 p53 gene being described, Figure 39 (SEQ ID NO:215) presents only the coding sequence for the human wild type p53 gene. The start codon is at position 1 and the stop codon is at position 1180.

Plasmid Cx22A, containing a mutant form of the human 35 p53 gene, was received from Arnold Levine (Princeton University, Princeton, New Jersey). Relative to the wild type p53 sequence presented in Figure 39, the G at nucleotide position 524 is substituted with an A,

changing the arg amino acid at codon 175 of the wild type protein to a his amino acid in Cx22A.

Plasmid pMM110 (Example 15, Figure 18) contains the vaccinia H6 promoted wildtype human p53 gene in the ALVAC 5 C5 insertion site. The human p53 gene contains two PflmI sites. p53 coding sequences upstream from the first PflmI site and downstream from the second PflmI site are the same in pMM110 as in Cx22A. pMM110 was digested with PflmI to remove the 853 central base pairs of the p53 10 gene. The 853bp PflmI fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM143, contains the H6 promoted mutant p53 gene.

Recombination between donor plasmid pMM143 and ALVAC 15 rescuing virus generated recombinant virus vCP270. vCP270 contains the mutant form of the human p53 gene under the control of the vaccinia H6 promoter in the C5 locus.

Plasmid pR4-2 containing a mutant form of the human 20 p53 gene was received from Arnold Levine (Princeton University, Princeton, New Jersey). Relative to the wild type p53 sequence presented in Figure 39, the G at nucleotide position 818 is substituted by an A, changing the arg codon at amino acid position 273 to a his codon 25 in pR4-2.

Plasmid pMM110 (Example 15, Figure 18) contains the vaccinia H6 promoted human wildtype p53 gene in the ALVAC C5 insertion site. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream 30 from the second PflmI site are the same in pMM110 as in pR4-2. pMM110 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from pR4-2 containing the base change at nucleotide position 818 was inserted. The resultant 35 plasmid, pMM144, contains the H6 promoted mutant form of the human p53 gene in the C5 insertion locus.

Recombination between donor plasmid pMM144 and ALVAC

rescuing virus generated recombinant virus vCP269. vCP269 contains the mutant form of the human p53 gene under the control of the vaccinia H6 promoter in the C5 locus.

5 Mutant forms of Human p53 into NYVAC.

Plasmid Cx22A, described above, contains a mutant form of the human p53 gene, in which the G at nucleotide position 524 (Figure 39) is substituted by an A, changing the arg codon at amino acid position 175 to a his codon 10 in Cx22A.

Plasmid pMM106 (Example 15) contains the vaccinia H6 promoted wild-type human p53 gene in the NYVAC I4L insertion locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from 15 the second PflmI site are the same in pMM106 as in Cx22A. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from Cx22A containing the base change at position 524 was inserted. The resultant plasmid, pMM140, contains the H6 20 promoted mutant p53 gene.

Recombination between donor plasmid pMM140 and NYVAC rescuing virus generated recombinant virus vP1234. vP1234 contains the mutant form of the human p53 gene under the control of the vaccinia H6 promoter in the I4L 25 locus.

Plasmid pR4-2, described above, contains a mutant form of the human p53 gene, in which the G at nucleotide position 818 (Figure 39) is substituted by an A, changing the arg codon at amino acid position 273 to a his codon 30 in pR4-2.

pMM106 (Example 15) contains the H6 promoted wild-type human p53 gene in the I4L locus. p53 coding sequences upstream from the first PflmI site and p53 coding sequences downstream from the second PflmI site 35 are the same in pMM106 as in pR4-2. pMM106 was digested with PflmI to remove the 853 central base pairs of the p53 gene. The 853bp PflmI fragment from pR4-2 containing

the base change at position 818 was inserted. The resultant plasmid, pMM141, contains the H6 promoted mutant p53 gene.

Recombination between donor plasmid pMM141 and NYVAC 5 rescuing virus generated recombinant virus vP1233. vP1233 contains the mutant form of the human p53 gene under the control of the vaccinia H6 promoter in the I4L locus.

A listing of the wildtype and mutant forms of murine 10 p53 and the mutant forms of human p53 present in ALVAC and NYVAC recombinants described in Examples 31 and 32 is provided in Table 29.

Table 29

	<u>Recombinant Virus</u>	<u>Parent Virus</u>	<u>Species</u>	<u>Gene Insert</u>
	vCP263	ALVAC	murine	w.t. p53
20	vCP267	ALVAC	murine	p53 (+3 aa)
	vCP270	ALVAC	human	p53 (aa 175; R to H)
	vCP269	ALVAC	human	p53 (aa 273; R to H)
25	vP1234	NYVAC	human	p53 (aa 175; R to H)
	vP1233	NYVAC	human	p53 (aa 273; R to H)

30 Immunoprecipitation.

ALVAC and NYVAC based recombinants vP1101, vP1096, vP1098, vCP207, vCP193, vCP191 (all described in Example 15; Table 22, as well as ALVAC and NYVAC based recombinants vCP270, vCP269, vP1233, vP1234 described in 35 this Example, Table 29), contain wild type or mutant forms of the human p53 gene. All of these recombinant virus were assayed for expression of the human p53 gene using immunoprecipitation.

Recombinant or parental virus were inoculated onto 40 preformed monolayers of tissue culture cells in the presence of radiolabelled ³⁵S-methionine and treated as previously described (Taylor et al., 1990).

Immunoprecipitation reactions were performed using a human p53 specific monoclonal antibody 1801. A protein of between 47 and 53 kDa was precipitated from cells infected with any of the recombinant viruses, vP1101, 5 vP1096, vP1098, vCP207, vCP193, vCP191, vCP270, vCP269, vP1233, or vP1234, but not from uninfected cells or cells infected with parental ALVAC or NYVAC virus.

Based upon the properties of the poxvirus vector systems, NYVAC, ALVAC and TROVAC cited above, such 10 vectors expressing either wildtype or mutant forms of p53 provide valuable reagents to determine whether endogenous CTL activities can be detected in patient effector populations (TILs, PBMC, or lymph node cells); and, valuable vehicles for the stimulation or the augmenting 15 of such activities; for instance, augmenting such activities by *in vitro* or *ex vivo* stimulation with these recombinant viruses. Further, the highly attenuated properties of both NYVAC and ALVAC allow the recombinants of the invention to be used for interventive 20 immunotherapeutic modalities discussed above, e.g., *in vivo* interventive immunotherapy

Example 33 - ERB-B-2 INTO COPAK

Plasmid ErbB2SphIstop was obtained from Jeffrey Marks (Duke University Center). ErbB2SphIstop contains a 25 3.8 kb human erb-B-2 cDNA insert cloned in pUC19. The insert extends from nt 150 through nt 3956 (Yamamoto et al., 1986) and contains the entire erb-B-2 coding sequence. In ErbB2SphIstop, the SphI site at nt 2038 was mutagenized by the addition of an XbaI linker, creating 30 an in frame stop codon. The remaining, truncated, ORF thus specifies an extracellular, secretable form of the erb-B-2 gene product, mimicking the translation product of the 2.3 kb mRNA. Plasmid ErbB2SphIstop was digested with XhoI and the 3.8 kb erb-B-2 fragment was isolated. 35 This isolated fragment was ligated with COPAK vector plasmid pSD555 cut with XhoI, resulting in plasmid pMM113.

Plasmid pSD555 was derived as follows. Plasmid pSD553 (Example 17) is a vaccinia deletion/insertion plasmid of the COPAK series. It contains the vaccinia K1L host range gene (Gillard et al., 1986) within 5 flanking Copenhagen vaccinia arms, replacing the ATI region (orfs A25L, A26L; Goebel et al., 1990).

Plasmid pSD553 was cut with NruI and ligated with a SmaI/NruI fragment containing the synthetic vaccinia H6 promoter element (Perkus et al., 1989) upstream from the 10 NruI site located at -26 relative to the translation initiation codon. The resulting plasmid, pMP553H6, contains the vaccinia H6 promoter element located downstream from the K1L gene within the A26L insertion locus.

15 To complete the vaccinia H6 promoter and add a multicloning region for the insertion of foreign DNA, plasmid pMP553H6 was cut with NruI/BamH1 and ligated with annealed synthetic oligonucleotides MPSYN349 (SEQ ID NO:216) 5'
20 CGATATCCGTTAACGTTGATCGTAATGGAGCTCCTGCAGCCCCGGGG 3' and MPSYN350 (SEQ ID NO:217) 5'
GATCCCCCGGGCTGCAGGAGCTCCATTACGATAACAAACTAACGGATATCG 3'. The resulting plasmid, pSD555, contains the entire H6 promoter region followed by a multicloning region.

25 Recombination between donor plasmid pMM113 and NYVAC rescuing virus generated recombinant virus vP1100. vP1100 contains the erb-B-2 gene under the control of the vaccinia H6 promoter in the I4L locus, along with the vaccinia K1L host range gene.

30 Immunoprecipitation. Preformed monolayers of Vero cells were inoculated at 10 pfu per cell with parental NYVAC virus and recombinant virus vP1100 in the presence of radiolabelled ³⁵S-methionine and treated as previously described (Taylor et al., 1990). Immunoprecipitation 35 reactions were performed using a human erb-B-2 specific monoclonal antibody TA1-1C. A protein of approximately 97 kDa was precipitated from cells infected with vP1100,

but not from uninfected cells or cells infected with parental NYVAC virus.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

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WHAT IS CLAIMED IS:

1. A modified recombinant virus, said modified recombinant virus having virus-encoded genetic functions inactivated therein so that the virus has attenuated 5 virulence, yet retained efficacy; said virus further comprising exogenous DNA in a nonessential region of the virus genome, said exogenous DNA encoding at least one cytokine and/or a tumor associated antigen.
2. The virus of claim 1 wherein said virus is a 10 poxvirus.
3. The virus of claim 2 wherein the poxvirus is a vaccinia virus.
4. The virus of claim 3 wherein the genetic functions are inactivated by deleting at least one open 15 reading frame.
5. The virus of claim 4 wherein the deleted genetic functions include a C7L-K1L open reading frame, or, a host range region.
6. The virus of claim 5 wherein at least one 20 additional open reading frame is deleted; and, the additional open reading frame is selected from the group consisting of: J2R, B13R + B14R, A26L, A56R, and I4L.
7. The virus of claim 5 wherein at least one 25 additional open reading frame is deleted; and, the additional open reading frame is selected from the group consisting of: a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, and a large subunit, ribonucleotide reductase.
8. The virus of claim 6 wherein J2R, B13R + B14R, 30 A26L, A56R, C7L - K1L and I4L are deleted from the virus.
9. The virus of claim 7 wherein a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range region, and a large subunit, ribonucleotide reductase are deleted from 35 the virus.
10. The virus of claim 8 which is a NYVAC recombinant virus.

11. The virus of claim 9 which is a NYVAC recombinant virus.

12. The virus of claim 11 wherein the exogenous DNA codes for at least one of: human tumor necrosis factor; 5 nuclear phosphoprotein p53, wildtype or mutant; human melanoma-associated antigen; IL-2; IFN γ ; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen.

13. The virus of claim 12 which is vP1200, vP1101, vP1098, vP1239, vP1241, vP1237, vP1244, vP1243, vP1248, 10 NYVAC+IFN γ +IL-2, vP1250, vP1246, NYVAC+IL-12, vP1230, vP1245, NYVAC+IFN γ +B7, vP1234, vP1233, vP1100 or vP1096.

14. A modified recombinant avipox virus which is modified so that it has attenuated virulence in a host; and, which contains exogenous DNA in a 15 nonessential region of the virus genome, said exogenous DNA encoding at least one cytokine and/or a tumor associated antigen.

15. The virus of claim 14 wherein said virus is a canarypox virus.

20 16. The virus of claim 15 which is an ALVAC recombinant virus.

17. The virus of claim 16 wherein the exogenous DNA codes for at least one of: human tumor necrosis factor; nuclear phosphoprotein p53, wildtype or 25 mutant; human melanoma-associated antigen; IL-2; IFN γ ; IL-4; GMCSF; IL-12; B7; erb-B-2 and carcinoembryonic antigen.

18. The virus of claim 17 which is vCP245, vCP235, vCP207, vCP193, vCP275, vCP277, vCP271, vCP278, 30 vCP275+IFN γ , vCP277+IFN γ , ALVAC+IL-4, vCP290, vCP285, ALVAC+IL-12, vCP268, ALVAC+IFN γ +B7, vCP263, vCP267, vCP270, vCP269, or vCP191.

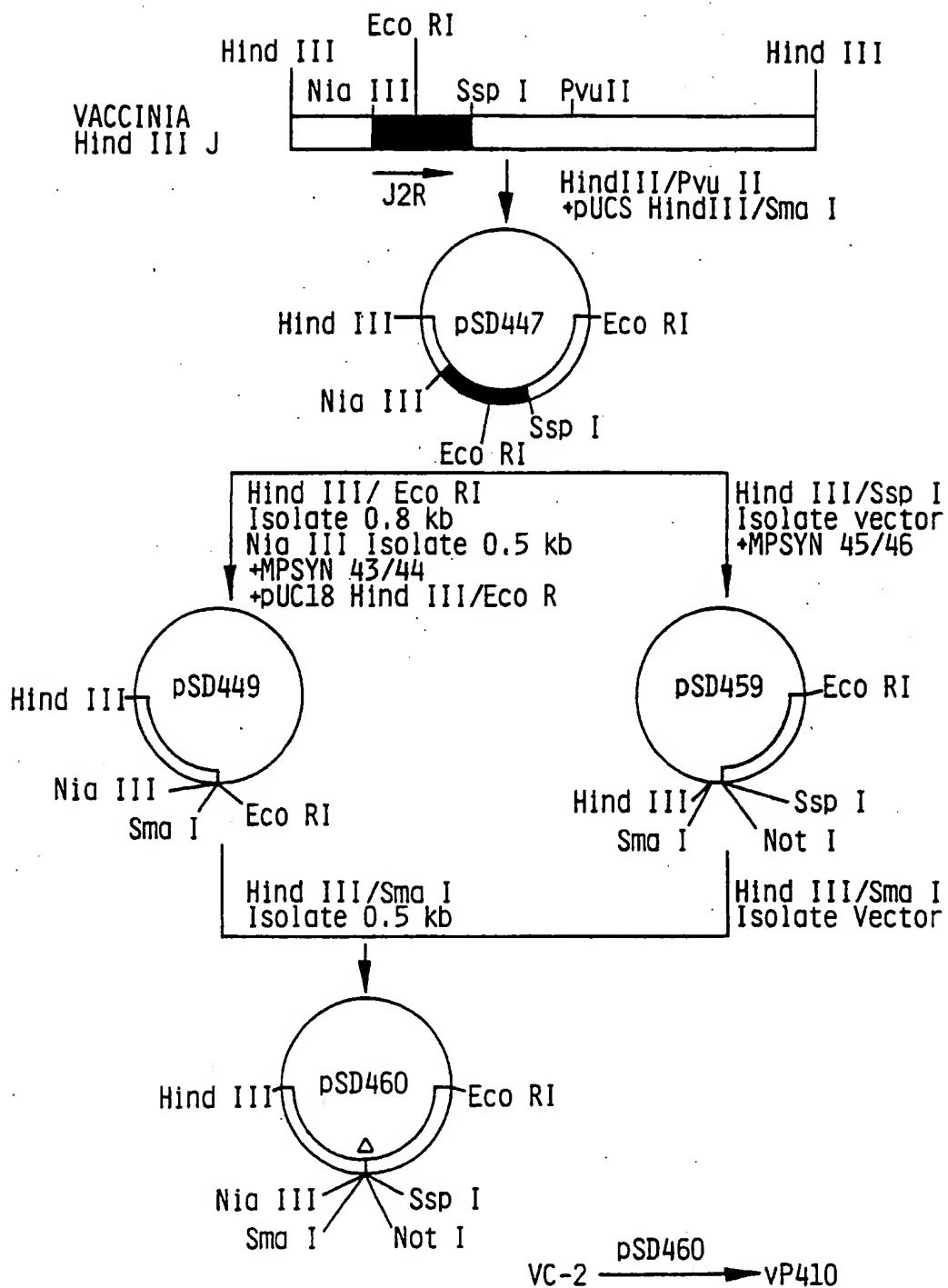
19. A method for treating a patient in need of cancer treatment comprising administering to said 35 patient a composition comprising a virus as claimed in any one of claims 1, 12, 14 or 17 in admixture with a suitable carrier.

20. A composition for inducing an antigenic or immunological response comprising a virus as claimed in any one of claims 1, 12, 14 or 17 in admixture with a suitable carrier.

5 21. A method for expressing a gene product in a cell cultured *in vitro* comprising introducing into the cell a virus as claimed in any one of claims 1, 12, 14 or 17.

22. A cytokine and/or tumor associated
10 antigen prepared from *in vitro* expression of a virus as claimed in any one of claims 1, 12, 14 or 17.

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**FIG. 1**

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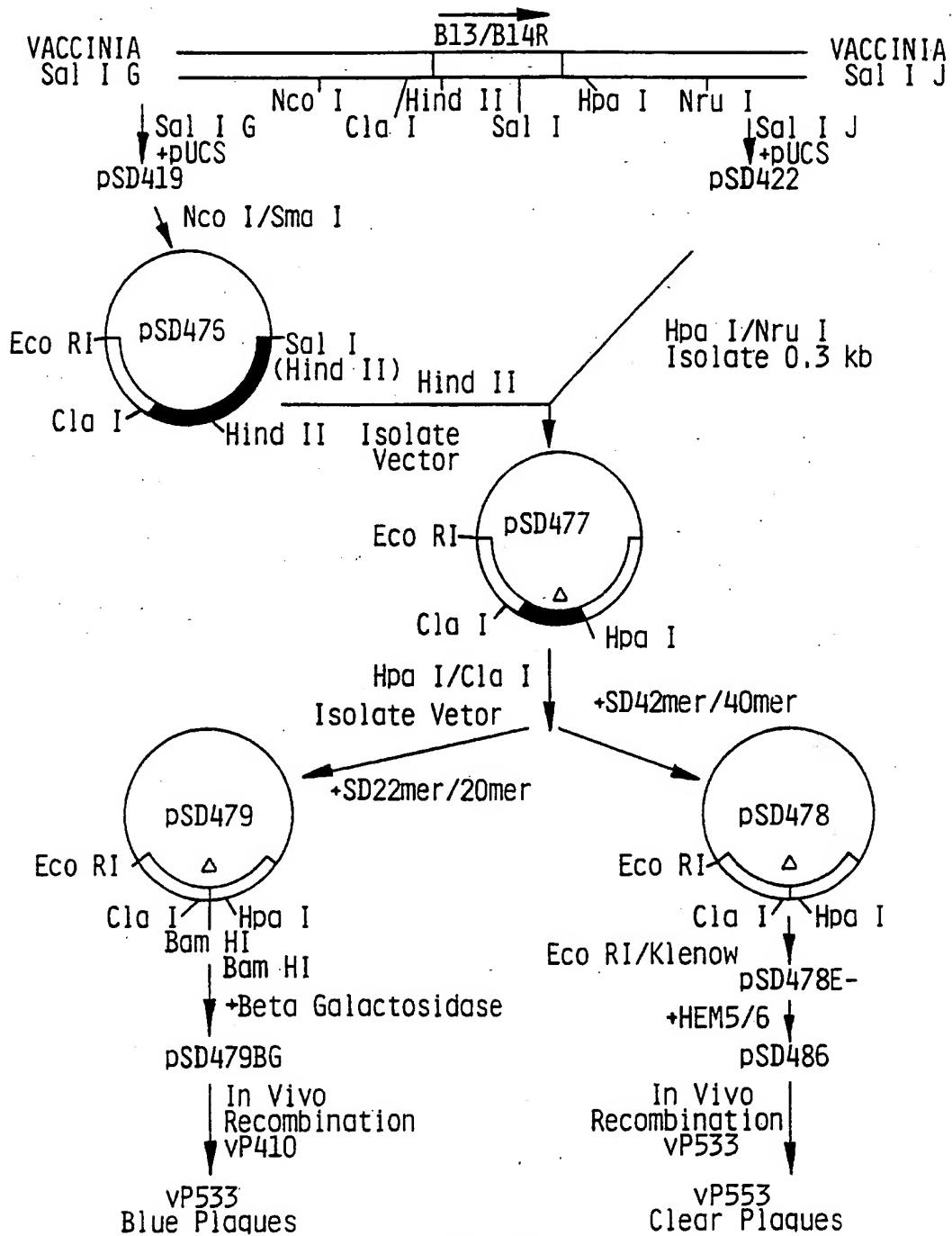


FIG.2

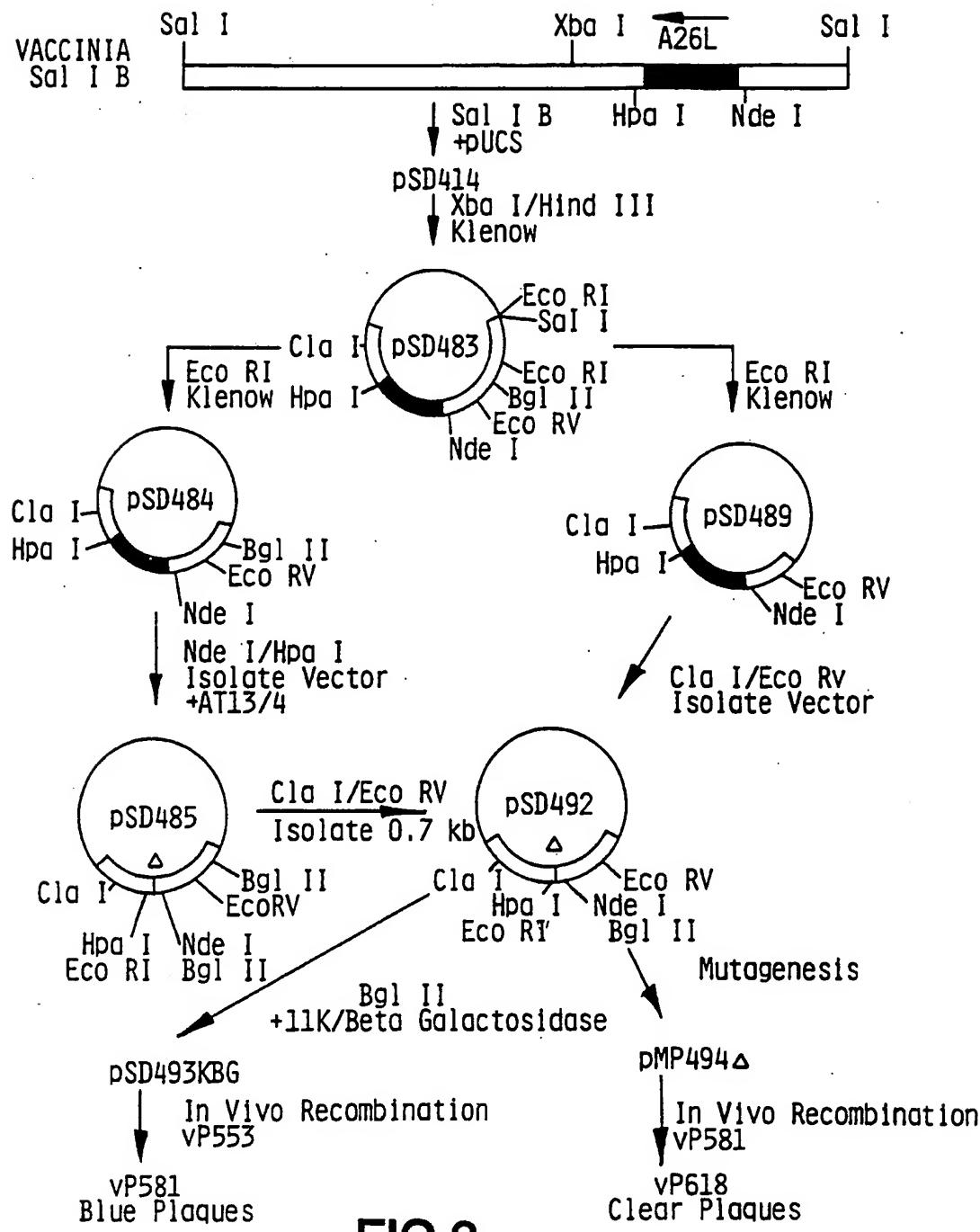


FIG.3

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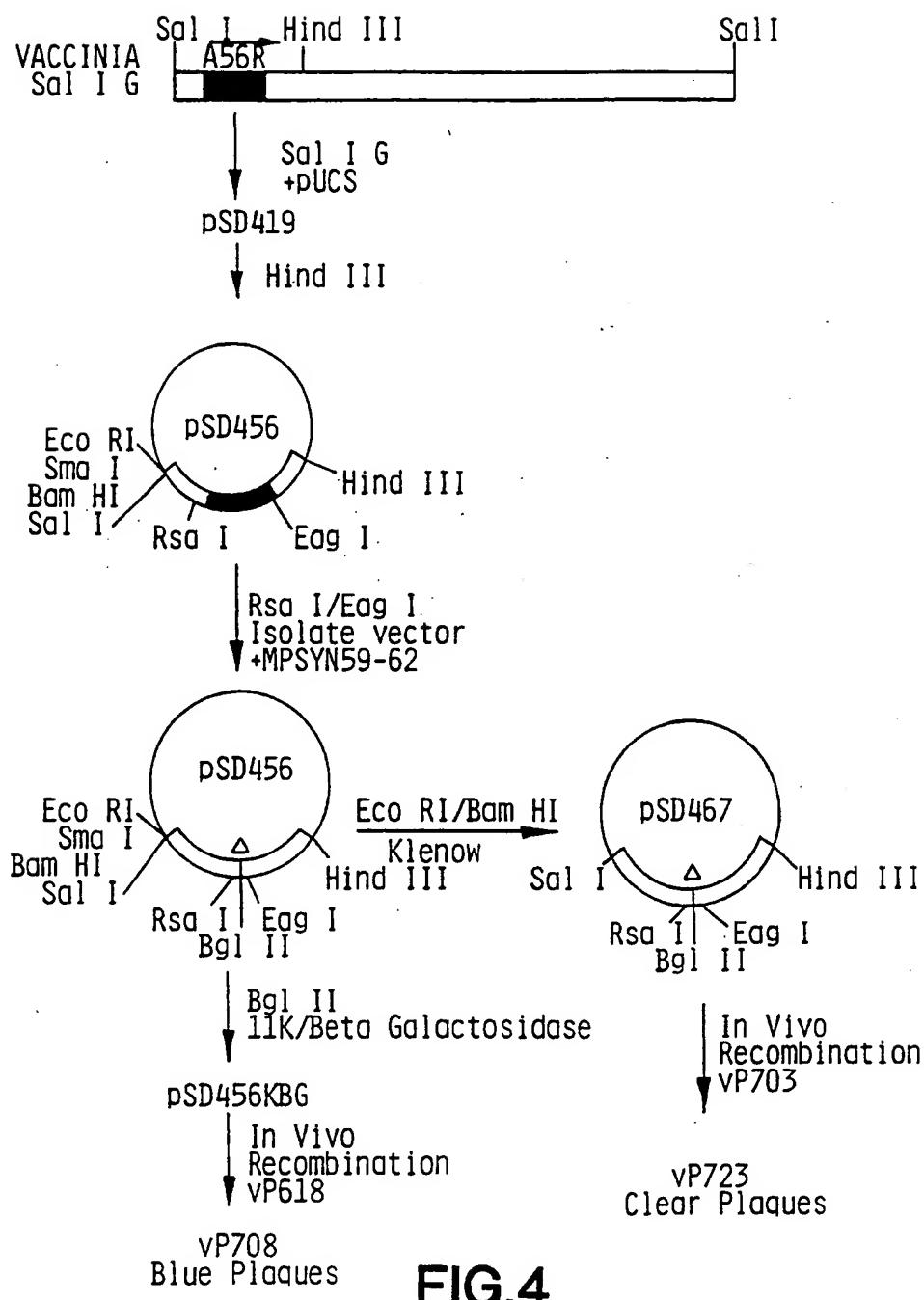
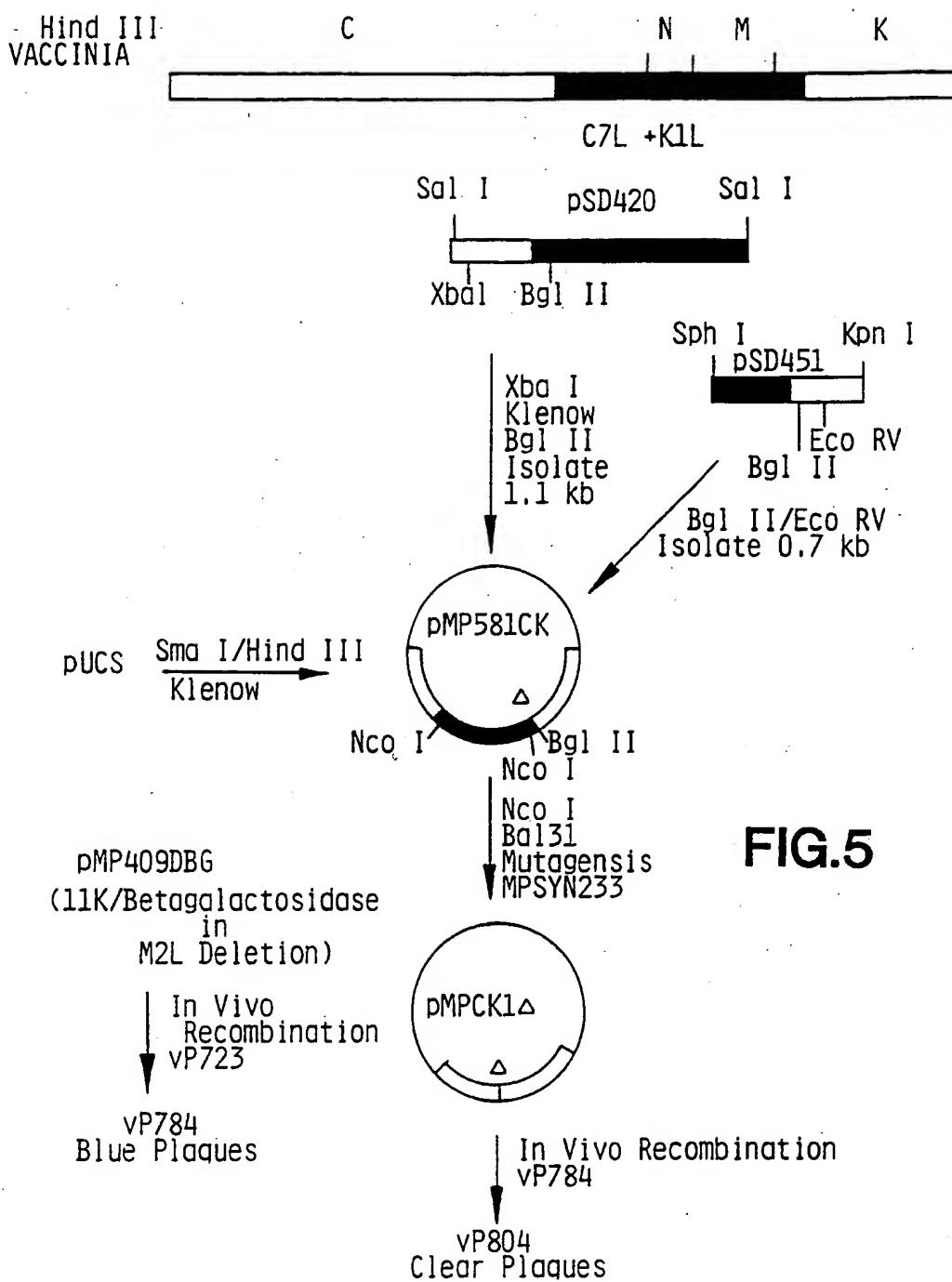


FIG.4

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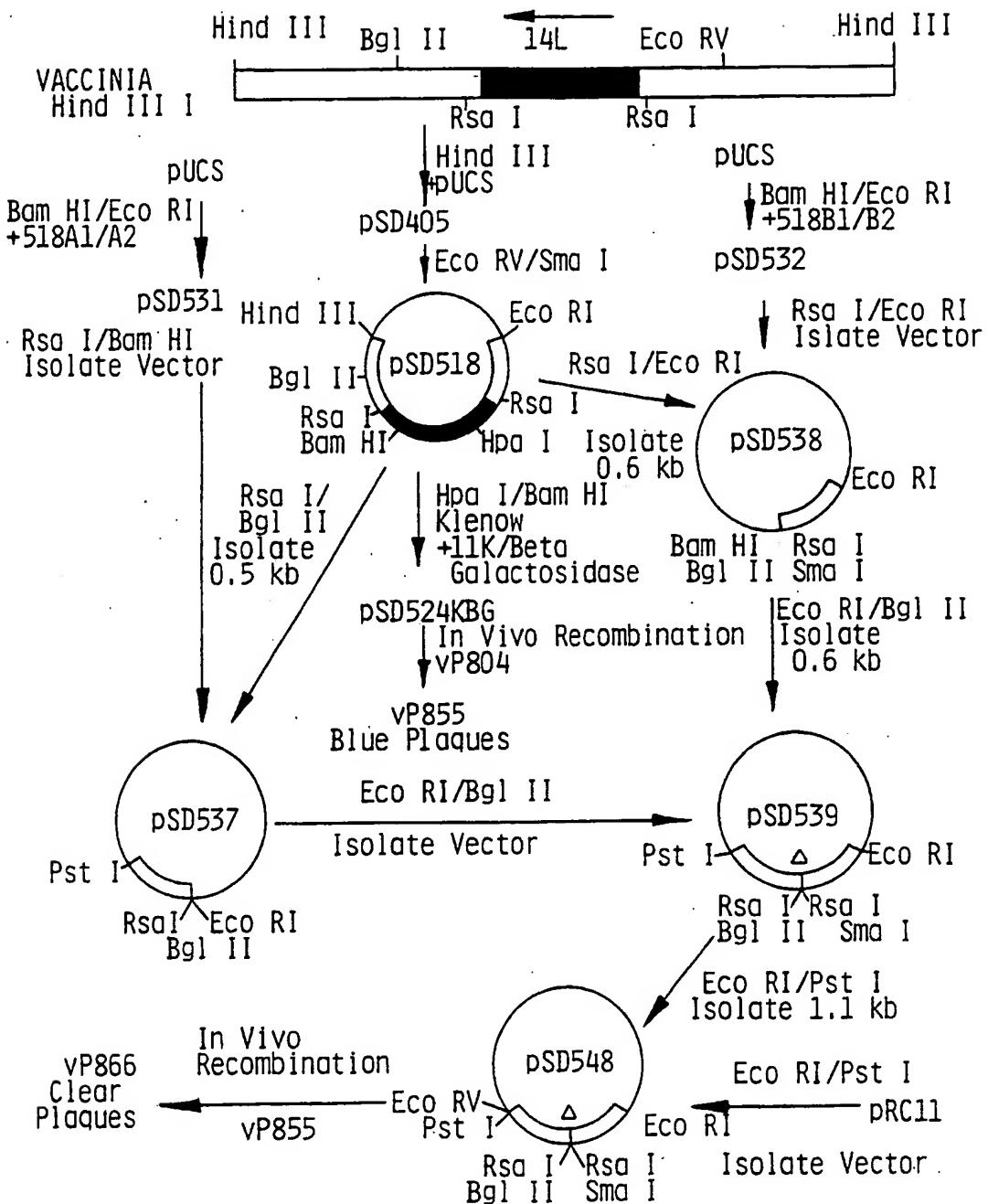
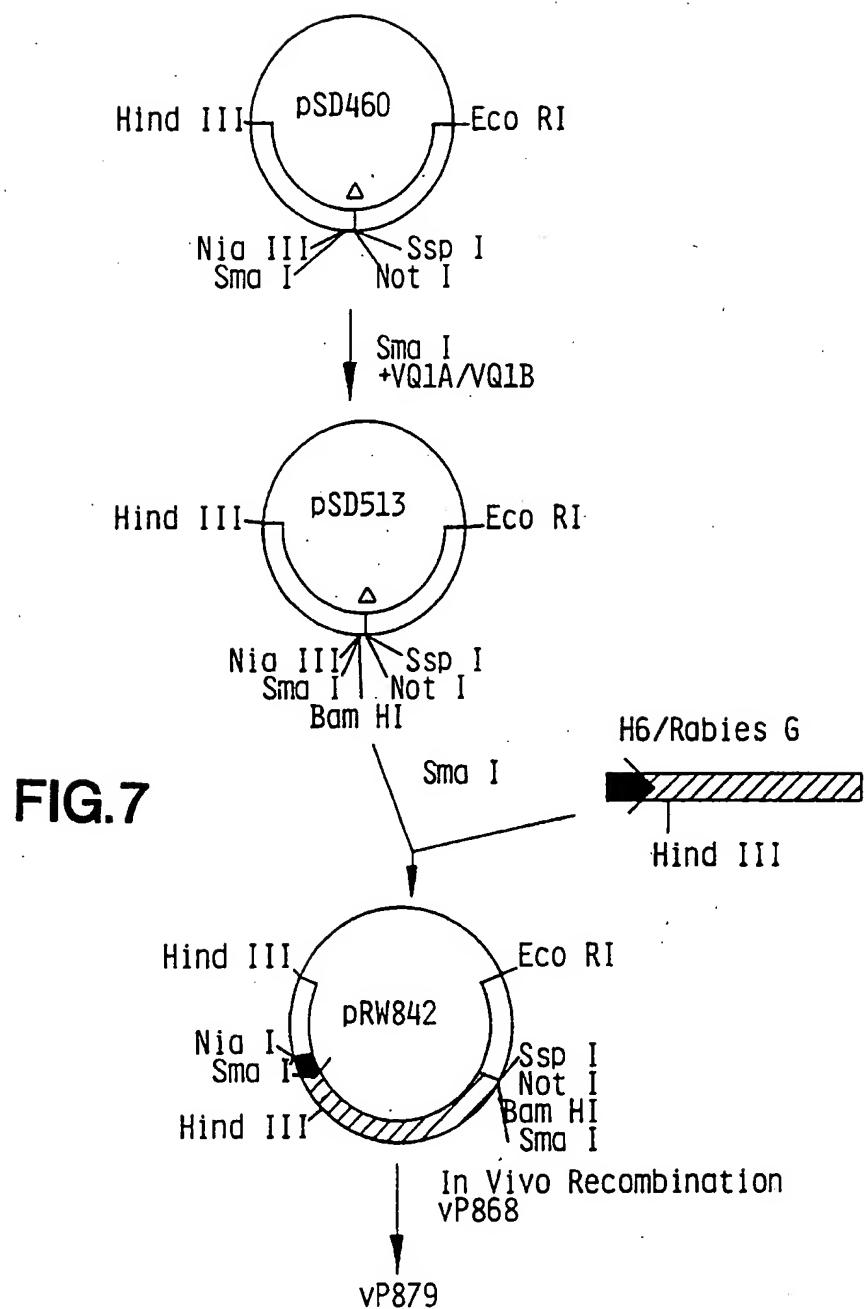


FIG.6

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1	TGAATGTTAA	ATGTTACT	TTGGATGAAG	CTATAAATAT	GCATTGGAAA	AATAATCCAT
61	TTAAAGAAAG	GATTCAAATA	CTACAAAACC	TAAGCGATAA	TATGTTAATC	AAGCTTATTTC
121	TTAACGACGC	TTTAAATATA	CACAAATAAA	CATAATTTTT	GTATAACCTA	ACAAATAACT
181	AAAACATAAA	AATAATAAA	GGAAATGTAA	TATCGTAATT	ATTTTACTCA	GGAATGGGGT
241	TAATATTTA	TATCACGTGT	ATATCTATAC	TGTTATCGTA	TACTCTTAC	AATTACTATT
301	ACGAATATGC	AAGAGATAAT	AAGATTACGT	ATTTAAGAGA	ATCTTGTAT	GATAATTGGG
361	TACGACATAG	TGATAATGC	TATTCGCAT	CGTTACATAA	AGTCAGTTGG	AAAGATGGAT
421	TTGACAGATG	TAACCTAATA	GGTCAAAAAA	TGTTAAATAA	CAGCATTCTA	TCGGAAGATA
481	GGATACCACT	TATATTATAC	AAAAAATCACT	GGTTGGATAA	AACAGATTCCT	GCAATTATTCG
541	TTAAAGATGA	AGATTACTGC	GAATTGGTAA	ACTATGACAA	TTAAAGGCCA	TTTATCTCAA
601	CGACATCGT	TAATTCTTCC	ATGTTTTATG	TATGTGTTTC	AGATATTATG	AGATTACTAT
661	AAACTTTTG	TATACCTATA	TTCCGTAAAC	TATATTAATC	ATGAAGAAAAA	TGAAAAAGTA
721	TAGAAGCTGT	TCACGAGCGG	TTGTTGAAA	CAACAAAATT	ATACATTCAA	GATGGCTTAC
781	ATATACGTCT	GTGAGGCTAT	CATGGATAAT	GACAATGCAT	CTCTAAATAG	TTTTTGGAC
841	AATGGATTCG	ACCTAACAC	GGAAATATGGT	ACTCTACAA	CTCCTCTTGA	AATGGCTGTA
901	ATGTTCAAGA	ATACCGAGGC	TATAAAAATC	TTGATGAGGT	ATGGAGCTAA	ACCTGTAGTT
961	ACTGAATGCA	CAACTTCTTG	TCTGCATGAT	GGGGTGTGAA	GAGACGACTA	CAAAATAGTG
1021	AAAGATCTGT	TGAAGAATAA	CTATGTAAC	AATGTTCTT	ACAGCGGAGG	CTTTACTCCT
1081	TTGTGTTGG	CAGCTTACCT	TAACAAAGTT	AATTGGTTA	AACTTCTATT	GGCTCATTG
1141	GCGGATGTAG	ATATTCAAA	CACGGATCGG	TTAACCTCCTC	TACATATAGC	CGTATCAAAT
1201	AAAATTAA	CAATGGTTAA	ACTTCTATTG	AACAAAGGTG	CTGATACTGA	CTTGCTGGAT
1261	AACATGGGAC	GTACTCCTT	AATGATCGCT	GTACAATCTG	GAAATATTGA	AATATGTAGC
1321	ACACTACTA	AAAAAAATAA	AATGTCCAGA	ACTGGGAAAAA	ATTGATCTTG	CCAGCTGTAA
1381	TTCATGGTAG	AAAAGAAGTG	CTCAGGCTAC	TTTCAACAA	AGGAGCAGAT	GTAAACTACA
1441	TCTTGAAG	AAATGGAAAA	TCATATACTG	TTTGGATT	GATTAAGAAGA	AGTTACTCTG
1501	AGACACAAAA	GAGGTAGCTG	AAGTGGTACT	CTCAAAATGC	AGAACGATGA	CTGCGAAGCA
1561	AGAAAGTAGAG	AAATAACACT	TTATGACTTT	CTTAGTTGTA	AAAAAGATAG	AGATATAATG
1621	ATGGTCATAA	ATAACTCTGA	TATTGCAAGT	AAATGCAATA	ATAAGTTAGA	TTTATTTAAA
1681	AGGATAGTTA	AAAATAGAAA	AAAAGAGTTA	ATTTGTAGGG	TTAAAATAAT	ACATAAGATC
1741	TTAAAATTAA	TAATATACGCA	TAATAATAAA	AATAGATTAT	ACTTATTAC	TTCAAGAGATA
1801	AAATTTAAGA	TATTTACTTA	TTTAACTTAT	AAAGATCTAA	AATGCATAAT	TTCTAAATAA
1861	TGAAAAAA	GTACATCATG	AGCAACCGT	TAGTATATT	TACAATGGAG	ATTAACGCTC
1921	TATACCGTT	TATGTTTATT	GATTCAAGATG	ATGTTTTAGA	AAAGAAAGTT	ATTGAATATG
1981	AAAACTTAA	TGAAGATGAA	GATGACGACG	ATGATTATTG	TTGTAATCT	TTTTAGATG
2041	AAGAAGATGA	CGCGCTAAAG	TATACATATGG	TTACAAAGTA	TAAGTCTATA	CTACTAATGG
2101	CGACTTGTG	AAGAAGGTAT	AGTATAGTGA	AAATGTTGTT	AGATTATGAT	TATGAAAAAC
2161	CAAATAAATC	AGATCCATAT	CTAAAGGTAT	CTCCTTGC	CATAATTCTA	TCTATTCTA
2221	GTTTAGAATA	CTTTCTTAA	TATTTGTTA	CAGCTGAAGA	CGAAAAAAAT	ATATCGATAA
2281	TAGAAGATTA	TGTTAACTCT	GCTAAATAAGA	TGAAATTGAA	TGAGTCTGTG	ATAATAGCTA
2341	TAATCAGAGA	AGTCTAAAAA	GGAAATAAAA	ATCTAACTGA	TCAGGATATA	AAAACATTGG
2401	CTGATGAAAT	CAACAAGGAG	GAACGTAA	TAGCTAAACT	ATTGTTAGAT	AGAGGGGCCA
2461	AAGTAAATT	CAAGGATGTT	TACGGTTCT	CAGCTCTCCA	TAGAGCTGCT	ATTGGTAGGA
2521	AACAGGATAT	GATAAAGCTG	TTAACGATC	ATGGAGCTGA	TGTAAACTCT	TTAACATTG
2581	CTAAAGATAA	TCTTATTAA	AAAAAATAAT	ATCACGTTA	GTAAATTAA	AATATATTAA
2641	TAACTCTATT	ACTAATAACT	CCAGTGGATA	TGAACATAAT	ACGAAGTTA	TACATTCTCA
2701	TCAAAATCTT	ATTGACATCA	AGTTAGATTG	TGAAAATGAG	ATTATGAAAT	TAAGGAATAC
2761	AAAATAGGA	TGTAAGAACT	TACTAGAATG	TTTTATCAAT	AATGATATGA	ATACAGTATC
2821	TAGGGCTATA	AACAATGAAA	CGATTAAGAA	TTATAAAAAT	CATTTCCCTA	TATATAATAC
2881	GCTCATAGAA	AAATTCTATT	CTGAAAGTAT	ACTAAGACAC	GAATTATTGG	ATGGAGTTAT
2941	AAATTCTTT	CAAGGATTCA	ATAATAAATT	GCCTTACGAG	ATTCACTACA	TTATACTGG
3001	GAATCTTAAT	AACCATGAAC	TAAAAAAAAT	TTTAGATAAT	ATACATTAAA	AAGGTTAAATA
3061	GATCATCTGT	TATTATAAGC	AAAGATGTT	GTTGCCAATA	ATATACAACA	GGTATTGTT
3121	TTTATTTTA	ACTACATATT	TGATGTTCAT	TCTCTTATA	TAGTATACAC	AGAAAATTCA
3181	TAATCCACTT	AGAATTCTA	GTTATCTAG			

FIG.8

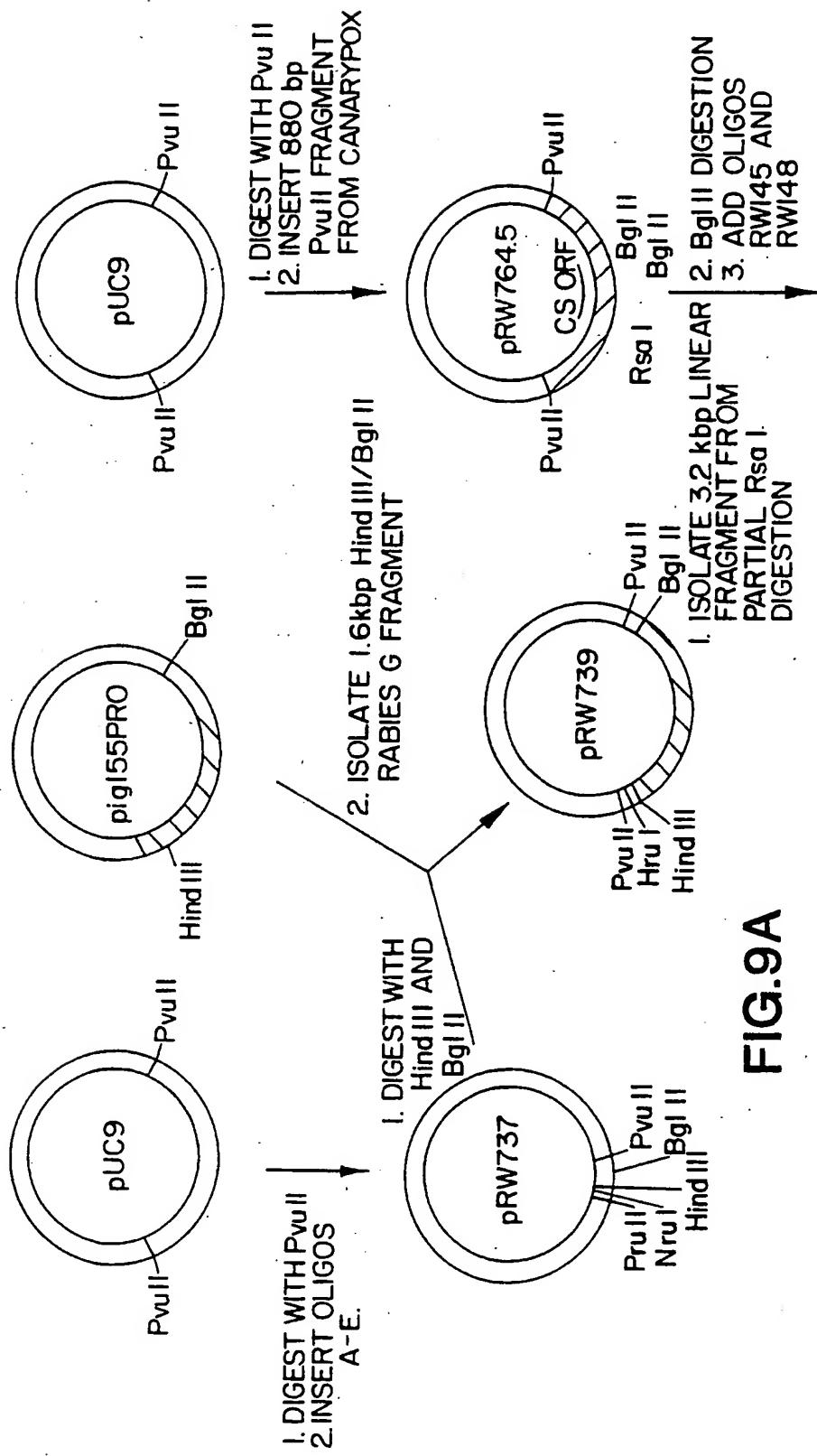
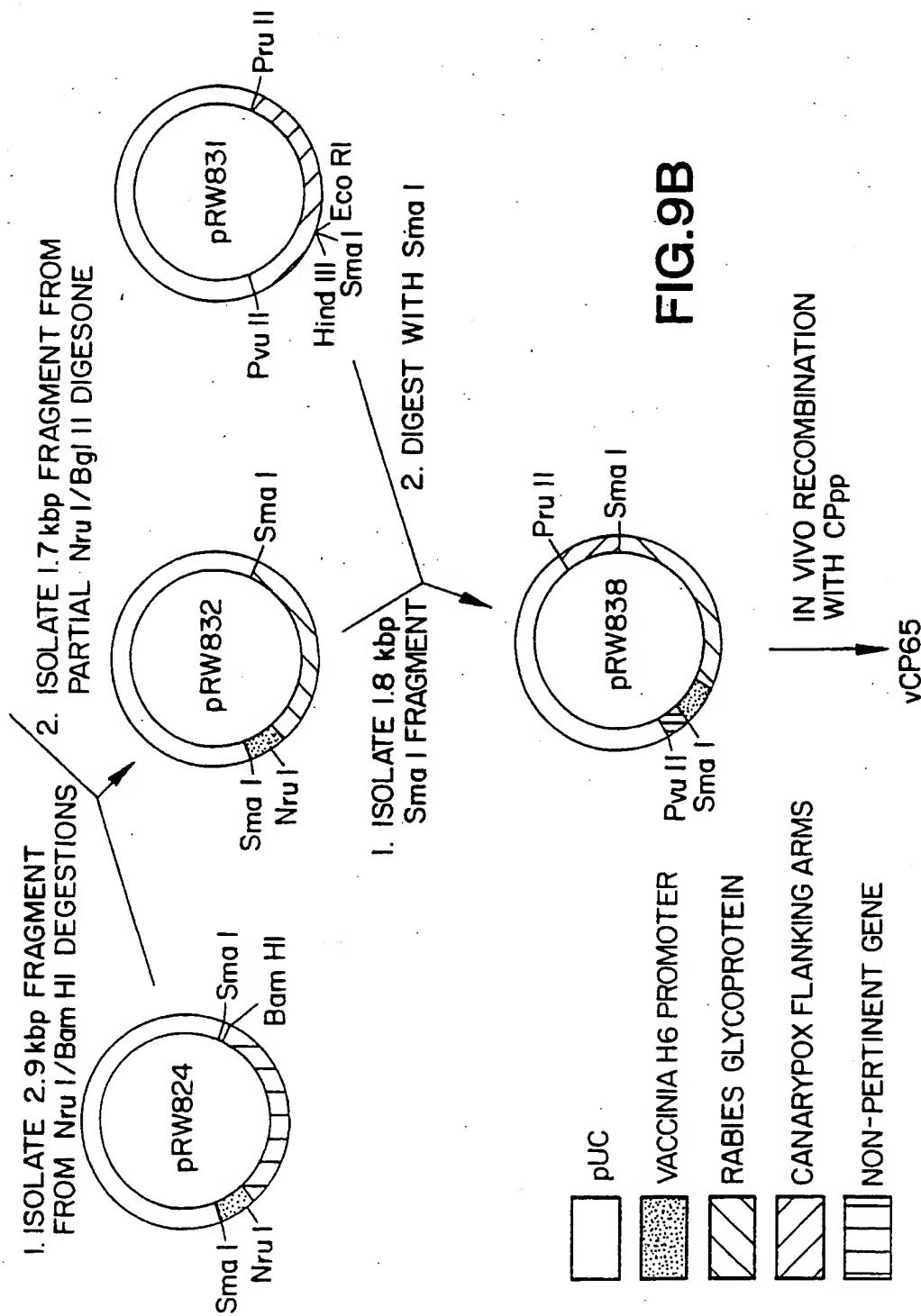


FIG. 9A

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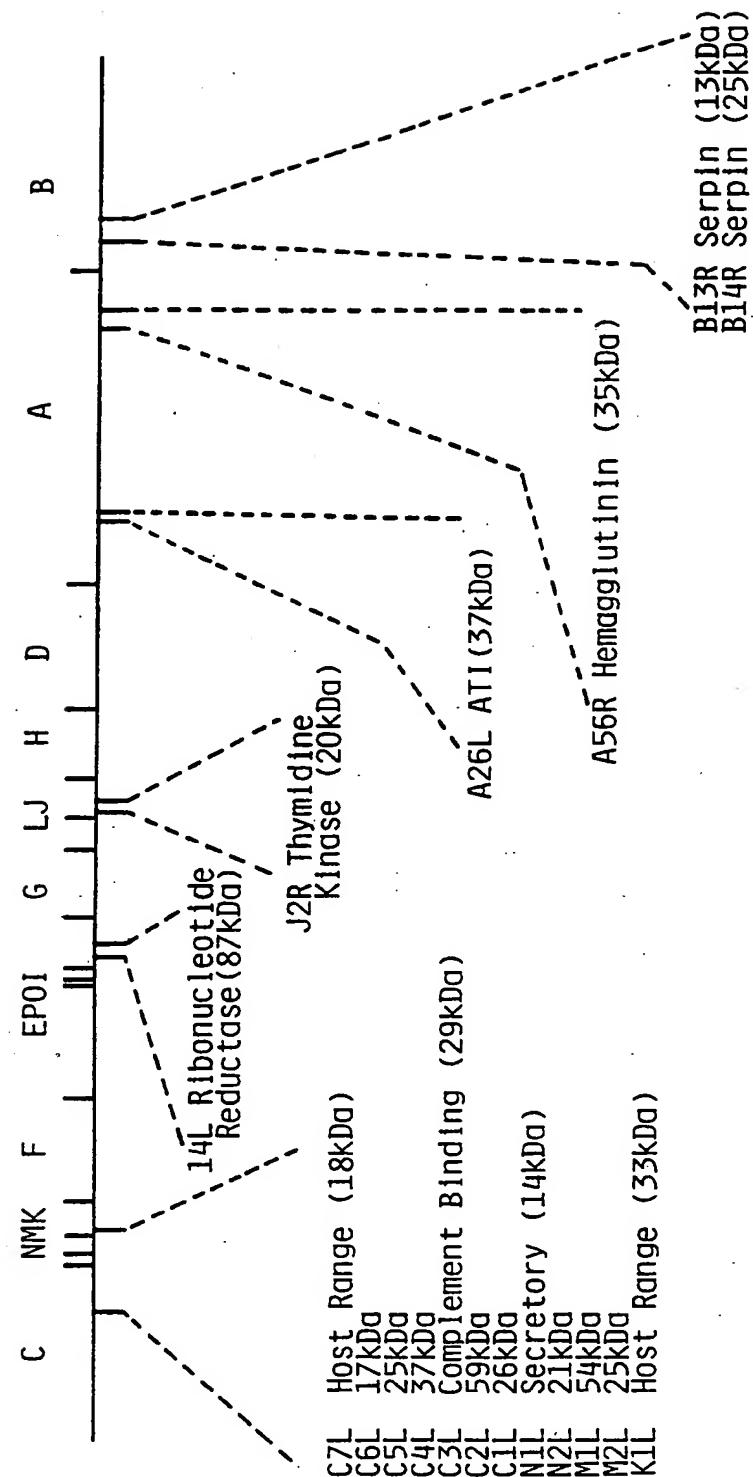


FIG. 10

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1 GATATCTGTG GTCTATAT ACTACACCC ACCGATATT ACCAACGAGT TTCTACAAG
 61 AAAACTTGT TAGTAGATAG AGATTCTTG ATTGTGTTA AAAGAAGTAC CAGTAAAAAG
 121 TGTGGCATAT GCATAGAAGA AATAAACAAA AAACATATT CCGAACAGTA TTTTGGATT
 181 CTCCCAAGTT GTAAACATAT TTTTGCTA TCATGTATAA GACGTTGGC AGATACTACC
 241 AGAAATACAG ATACTGAAAA TACGTGCTC GAATGTAGAA TAGTTTTTCC TTTCTATAA
 301 CCCAGTAGGT ATTGGATAGA TAATAAATAT GATAAAAAAA TATTATATAA TAGATATAAG
 361 AAAATGATT TTACAAAAAT ACCTATAAGA ACAATAAAA TATAATTACA TTTACGGAAA
 421 ATAGCTGGTT TTAGTTTAC AACTTAGAGT AATTATCATA TTGAATCTAT ATTGTTTTT
 481 AGTTATATAA AAACATGATT AGCCCCAAAT CGGATGAAAA TATAAAAGAT GTTGAGAATT
 541 TCGAATACAA CAAAAAGAGG AATCGTACGT TGTCATATC CAAACATATA AATAAAATT
 601 CAAAAGTAGT ATTACTGG ATGTTTAGAG ATCAACGTGT ACAAGATAAT TGGGCTTTAA
 661 TTTCACGCACA ACGATTAGCG TAAAAACTCA AAATACCTCT AAGAATATGC TTTTGTGCG
 721 TGCCAAAATT TCACACTACT ACTTCTAGAC ACTTTATGTT TTTAATATCC GGTCTAAAG
 781 AAGTCGCGGA AGAATGTAAA AGACTATGTA TAGGGTTTTC ATTGATATAT GGCGTACCAA
 841 AAGTAATAAT TCCGTGTATA GTAAAAAAAT ACAGAGTCGG AGTAATCATA ACGGATTTCT
 901 TTCCATTACG TGTTCCGAA AGATTAATGA AACAGACTGT AATATCTCTT CCAGATAACA
 961 TACCTTTTAC ACAAAGTAGAC GCTCATAATA TAGTACCTTG TTGGGAAGCT TCTGATAAAG
 1021 AAGAATACGG TGCAAGAACT TTAAGAAAAA AGATATTGAA TAAATTATAT GAATATATGA
 1081 CAGAATTTCG TGTTGTTCTG AAACATCCAT ACGGTCCATT TTCTATATCT ATTGCAAAC
 1141 CCAAAATAT ATCATTAGAC AAGACGGTAT TACCCGTAAA ATGGGCAACG CCTGGAACAA
 1201 AAGCTGGAAAT AATTGTTTTA AAAGAATTAA TAAAAAAACAG ATTACCGTCA TACGACGCGG
 1261 ATCATAACAA TCCTACGTG GACGCTTGA GTAACTTATC TCCGTGGCTA CATTGGTC
 1321 ATGTATCCGC ACAACGTGTT GCCTTAAAG TATTAATATG TATACGAGAA AGCAAAAAAA
 1381 ACGTTGAAAC GTTATAGAT GAAATAATG TAAGAAGAGA ACTATCGGAT AATTGGTT
 1441 ACTATAACAA ACATATTGAT AGTATCCAGT CTACTCATTCT ATGGGTTAGA AAAACATTAG
 1501 AAGATCACAT TAATGATCCT AGAAAGTATA TATATCCAT TAAACAACCTC GAAAAGCGG
 1561 AAACTCATGA TCCTCTATGG AACGCGTCAC AAATGCAGAT GGTGAGAGAA GGAAAGATGC
 1621 ATAGTTTTT ACGAATGTAT TGGGCTAAGA AGATACTTGA ATGGACTAGA ACACCTGAAG
 1681 ACGCTTTGAG TTATAGTATC TATTTGAACA ACAAGTACGA ACTAGACGGC ACGGATCCTA
 1741 ACGGATACGT AGGTTGTATG TGGTCTATT GCGGATTACA CGATAGACGG TGGAAAGCAA
 1801 GACCGATATT TGGAAAGATA AGATATATGA ATTATGAGAG TTCTAAGAAG AAATTGATG
 1861 TTGCTGTATT TATACAGAAA TACAATTAAG ATAATAATAA TACAGCATTG TAACCATCGT
 1921 CATCCGTTAT ACGGGGAATA ATATTACCAT ACAGTATTAT TAAATTTCT TACGAAAGAT
 1981 ATAGATCGGT ATTATCGTT AGTTTATTAA ACATTTATTA ATAAACATG TCTACTATTA
 2041 CCTGTTATGG AAATGACAAA TTTAGTTATA TAATTATGAA TAAAATTAAG ATAATAATAA
 2101 TGAAATCAA TAATTATGTA AATGCTACTA GATTATGTGA ATTACGAGGA AGAAAGTTA
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 2221 ATAAAACCTAA CCAGTTAAAAA ACGGATATGA TTATATACGT TAAGGATATT GATCATAAAG
 2281 GAAGAGATAC TTGCGGTTAC TATGTACACC AAGATCTGGT ATCTTCTATA TCAAATTGGA
 2341 TATCTCCGTT ATTGCGCGTT AAGGTAAATA AAATTATTAA CTATTATATA TGTAATGAAT
 2401 ATGATATACG ACTTAGCGAA ATGGAATCTG ATATGACAGA AGTAATAGAT GTAGTTGATA
 2461 AATTAGTAGG AGGATACAAT GATGAAATAG CAGAAATAAT ATATTGTT AATAAATTAA
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 2581 TTATAAATT TATAAATTAA AATAAAAT ACAAATACGA CATAAAGATA TTTATCTTT
 2641 AATTCTTGAT CTGAAAAAAC CATCTATAAA ACTAGATAAA AAGTTATTGAG ATAAAGATAA
 2701 TAATGAATCG AACGATGAAA AATTGGAAAC AGAAGTTGAT AGCTAATT TTTCTATCTA
 2761 AATAGTATT TAATGAA GTACGAAGT TTACGTTAGA TAAATAATAA AGGTCGATTT
 2821 TTACTTTGTT AAATATCAA TATGTCATTA TCTGATAAAAG ATACAAAAC ACACGGTGAT
 2881 TATCAACCAT CTAACGAACA GATATTACAA AAAATACGTC GGACTATGGA AAACGAAGCT
 2941 GATAGCCTCA ATAGAAGAAG CATTAAAGAA ATTGTTGTAG ATGTTATGAA GAATTGGGAT
 3001 CATCCTCAAC GAAGAAATAG ATAAGTTCT AAACCTGGAAA AATGATACAT TAAACGATTT
 3061 AGATCATCTA AATACAGATG ATAATATTAAG GGAATCATA CAATGTCTGA TTAGAGAATT
 3121 TGCCTTAAAG AAGATCAATT CTATTATGTA TAGTTATGCT ATGGTAAAAC TCAATTCTGA
 3181 TAACGAACAT TGAAAGATAA AATTAAGGAT TATTGTTATAG AACTATTCT TAAAGACAAA
 3241 CGTGGTTATA AACAAAAGCC ATTACCCGGA TTGGAAACTA AAATACTAGA TAGTATTATA
 3301 AGATTTTAAAG AACATAAAAT TAATAGGTT TTATAGATTG ACTTATTATA TACAATATGG
 3361 ATAAAAGATA TATATCAACT AGAAAGTTGA ATGACGGATT CTTAATTGAA TATTATGATT
 3421 CAATAGAAAT TATTGTCATG TCGTGTAAATC ATTTTATAAA TATATCAGCG TTACTAGCTA
 3481 AGAAAAACAA GGACTTTAAT GAATGGCTAA AGATAGAATC ATTTAGAGAA ATAATAGATA
 3541 CTTTAGATAA AATTAATTAC GATCTAGGAC AACGATATTG TGAAGAACCT ACGGCGCATC
 3601 ACATTCCAGT GTAATTATTG AGGTCAAAGC TAGTAACCTA ATAGATGACA GGACAGCTG

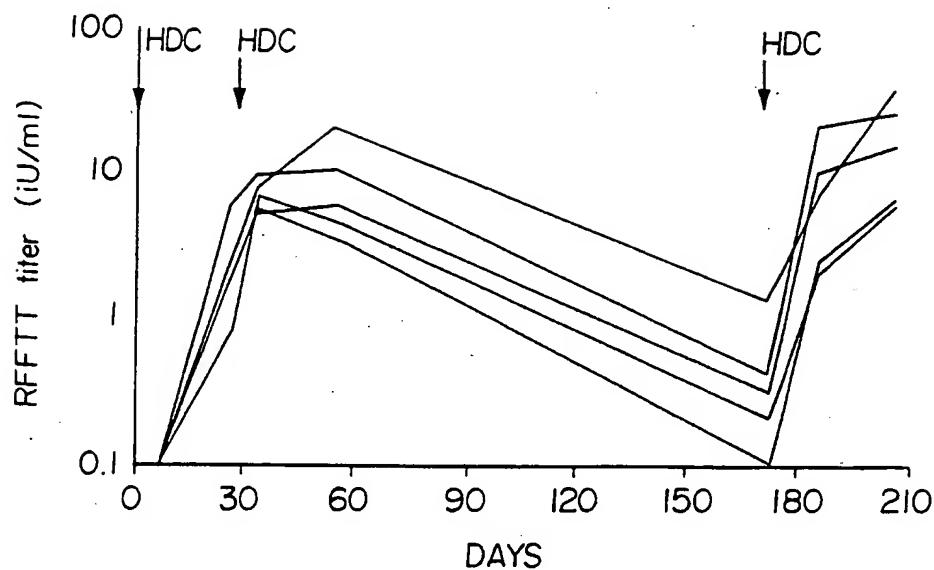
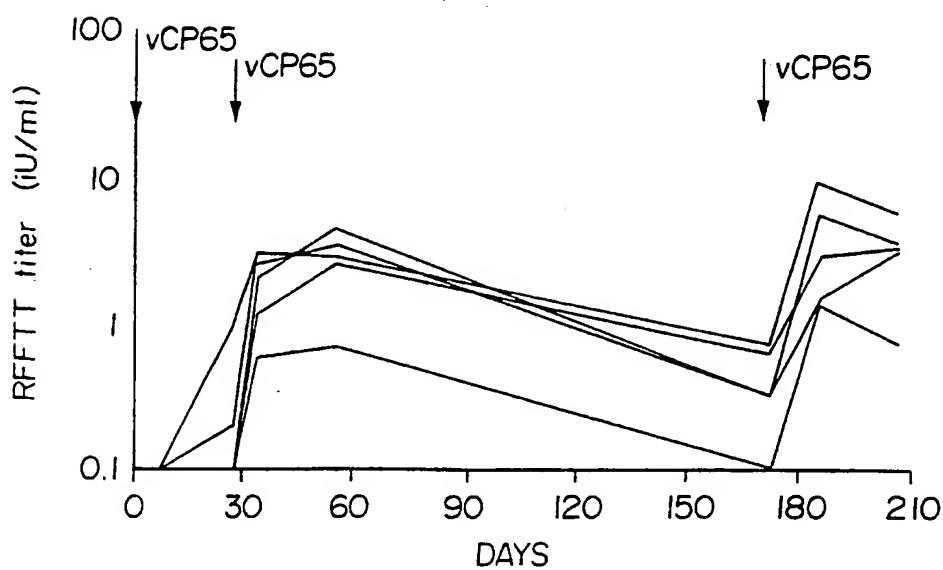
FIG. 11

SUBSTITUTE SHEET (RULE 26)

1 TGTCTGGACT AACTGATTC ATGGAACAAT TTTCATCAA AATATCAGTT ATACCTAGTT
 61 CTACAAAGAC AGAACTTGA TGTTATGTT GTGTTTGTAT AGAAAATTG GGGATACTAA
 121 CTGATATTTC TGAATATTTC TGAATATTTC ATGTTACTTA CTTACTCCTA TCTTAGACGA
 181 TAATAAAATT CGAGGCCTAA TATGTTTTC CAAATATTG AAATTCTTAT ACGTATCGGC
 241 GAAGAAAAGT AACATACAT AAGTGTATG CAAGTAAGGT ATGTTAATGA TATTGGATT
 301 AATTTCATTG ACAATACATA TGTCACAAACA TTCCACTCGT AATTATGTAC GGAACGACTT
 361 TAGTTAAATA CTTAGTCACA AAAAACTTAT GACTGTCATT ATCTGAAAAC GGTGATTCCC
 421 ATAATTCAGA ATACCTAATA TAAATAGAA TGCTCGCTTC TGGAGGTTTC CGGATACTAG
 481 ATAACATATC TTCTGTATTA TAGTTAATT CACTCATTTT ATTACATAAT ACAGTAACAT
 541 CTCCCGAAAC CAATGATGTT ATATTAGATT TACTTACATA CTTCTTGAA CTATCATGAA
 601 TACGTTGTT GATGTCATA AAGAAGATGG ATGTTATTC TGTTCTAGAT AGCAAGTTCT
 661 TTAAGTTATT CTTTGTCTGT ATTACTATCA TCGTCTTCAT CATCGTCTAA AGGTAGCATT
 721 ATATAATAAA TCTAATAGTT GATTTCCTGA TCTATCAGTA CTCGCTTCA ATAACATT
 781 TACTATAAGC ATAATAGAAG GCGGTGATAT CACTATTT TTATCGGGTA TTCTTTAGT
 841 AATTAGTTAG TTCGTTAGAAT TTCGTTAGAGA TAAAAGCCAA TTGTTGTTG ATACTGCTTA
 901 CGTTACTCAT GTTTCTTGT TCTGTTAATT AACAGGTATA CCCTTACAAAT AAGTTAATT
 961 AACCTTTAGG TTTTGTGAA GAACTTTAG CTTCTAGTTC CCTTATCCAT AATTGGGTCT
 1021 TAGATCTAGA TTCTTCCCCT GTATAAAGGG GGACATACCC AAAATCTTAA AATGTTTGT
 1081 CCGTTTCTAT AGTAAATGTC GTACATTCT TAATCAAAGT ATAAGGATT AGTAAAGGCG
 1141 TGTAAGAAC AATAGGTGAT AGTAATACTC TAAACCTTT ATTAAATATTA GCGATAAAACC
 1201 TTAAACACCA TAAAGGAAGA CATGTATTCC GTAGATCCAT CCCTAATTGA TTAAAGAAAT
 1261 GCATGTTAAA ATCATGATAA TGTTCAGTAG GAGAGGTATC GTAACAGTAA TACACGTTAT
 1321 TGCAGAGAGG ACTATGTGAA CCATTTCTA TCATATTCT TGCTGCTAAA ATATGCATCC
 1381 AAGCTACGTT TCCTGCATAG ACTCTGCTAT GAAATACTT ATTAAATCTCA TATTATACA
 1441 TTTTCTGCT TTTATACGAT CTTCTGTATA AAGTTTCTAG TACTGGACAG TATTCTCCGA
 1501 AACACCGTAA TGGGCGTAGC GACAAGTGCA TAATCTAAGT CCTATATTAG ACATAGTACC
 1561 GTTAGCTTCT AGTATATATT TCTCAGATAA CTTGTTACT AAGAGGATAA GCCTCTTTAT
 1621 GGTTAGATTG ATAATACGTA TTCTCGTTTC TTCTCTATCAT CGCATCTCCG GAGAAAGTTA
 1681 GGACCTACCG CAGAATAACT ACTCGTATAT ACTAAGACTC TTACGCCGT ATACAGACAA
 1741 GAATCTACTA CGTTCTCGT TCCGTTGATA TTAACGTCA TTATAGAGTC GTTAGTAAAC
 1801 TTACCCGCTA CATCATTTT CGAAGCAATA TGAATGACCA CATCTGCTGA TCTAAGCGCT
 1861 TCGTCCAAAG TACTTTATT TCTAACATCT CCAATCACGG GAACTATCTT TATTATATTA
 1921 CATTTCCTA CAAGATCTAG TAACCATTGG TCGATTCTAA TATCGTAAAC ACGAACTTCT
 1981 TTTAAAGAG GATTGAAACA AGATAAGATT ATTTATAATG TGTCTACCTA AAAATCCACA
 2041 CCCTCCGGTT ACCACGTATA CTAGTGTACG CATTGTTAGT ATTAAACTATA TAAGACCAA
 2101 ATTATATTTC CATTTCCTGT TATATTATAC TATATAATAA AAACAAATAA ATATACGAAT
 2161 ATTATAAGAA ATTTAGAACA CGTTATTAAA GTATTGCCTT TTTTATTAAAC GGCCTGTTCT
 2221 TGTAATTGCC GTTTAGAATA GTCTTTATT ACTTTAGATA ACTCTTCTAT CATAACCGTC
 2281 TCCTTATTCC AATCTTCTTC AGAAGTACAT GAGTACTTAC CGAAGTTTAT CATCATAGAG
 2341 ATTATATATG AAGAAA

FIG.12

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**FIG. 13A****FIG. 13C**

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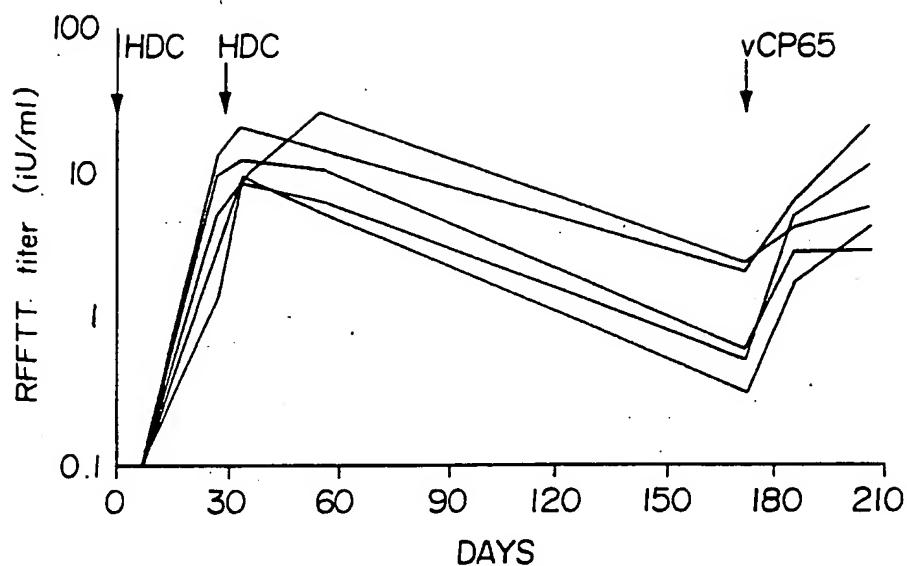


FIG. 13B

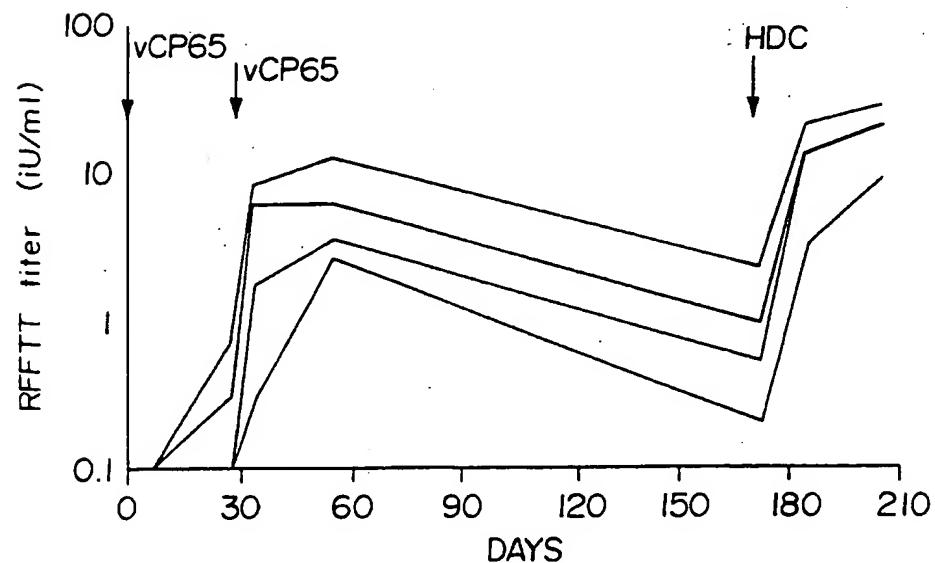


FIG. 13D

1 AGATATTGT TAGCTCTGC CGGAGATACC GTGAAAATCT ATTTCTGGA AGGAAAGGGA
 61 GGTCTTATCT ATTCTGTCAG CAGAGTAGGT TCCTCTAATG ACGAAGACAA TAGTGAATAC
 121 TTGCATGAAG GTCACTGTGT AGAGTTCAA ACTGATCATC AGTGTGTTGAT AACTCTAGCG
 181 TGACGAGTC CTCTAACAC TGTGGTTAT TGGCTGGAAT AAAAGGATAA AGACACCTAT
 241 ACTGATTATC TTTCATCTGT CAACGTTCT CTAAGAGATT CATAGGTATT ATTATTACAT
 301 CGATCTAGAA GTCTAATAAC TGCTAAGTAT ATTATTGGAT TTAACCGCGCT ATAAACCGCAT
 361 CCAAAACCTA CAAATATAGG AGAAGCTCT CTTATGAAAC TTCTTAAAGC TTTACTCTTA
 421 CTATTACTAC TCAAAAGAGA TATTACATTA ATTATGTGAT GAGGCATCCA ACATATAAAG
 481 AAGACTAAAG CTGTAGAAGC TGTTATGAAG AATATCTTAT CAGATATATT AGATGCATTG
 541 TTAGTTCTGT AGATCAGTAA CGTATAGCAT ACGAGTATAA TTATCGTAGG TAGTAGGTAT
 601 CCTAAATAAAT ATCTGATACA GATAATAACT TTGTAATTCAT ATTTCAGCAAT TTCTCTTAA
 661 TCATGATAAT TAAAGAGCAG CAGCGTGTG TTATTTTTG TTACGATAGT ATTTCCTAAAG
 721 TAAAGAGCAG GAATCCCTAG TATAATAGAA ATAATCCATA TGAAAAAATAT AGTAAATGTAC
 781 ATATTTCTAA TTGTTAACATA TTTATAGGT AATCCAGGAA GGGTAATTTT TACATATCTA
 841 TATACGCTTA TTACAGTTAT TAAAAAATATA CTTGCAAACA TGTTAGAAGT AAAAAAGAAA
 901 GAACTAATTT TACAAAGTGC TTACCAAAA TGCCAATGGG AATTACTTAG TATGTATATA
 961 ATGTATAAAAG GTATGAATAT CACAAACAGC AAATCGGCTA TTCCCAAGTT GAGAAACGGT
 1021 ATAATAGATA TATTTCTAGA TACCATTAAT AACCTTATAA GCTTGACGTT TCCTATAATG
 1081 CCTACTAAGA AAACTAGAAG ATACATACAT ACTAACGCCA TACGAGAGTA ACTACTCATC
 1141 GTATACTAC TGTGCTAAC AGTGACACTG ATGTTATAAC TCATCTTGTG TGTGGTATAA
 1201 ATGTATAATA ACTATATTAC ACTGGTTTT TATTTCTAGTT ATATACATA TAGTATTTAA
 1261 AATTATATTT GTATAATTAT ATTATTATAT TCAGTGTAGA AAGTAAAATA CTATAAATAT
 1321 GTATCTCTTA TTATTAACCTT ATTAGTAAAG TATGTTACTAT TCAGTTATAT TGTGTTATAA
 1381 AAGCTAAATG CTACTAGATT GATATAATG AATATGTAAT AAATTAGTAA TGTAGTATAC
 1441 TAATATTAAC TCACATTATG AATACTACTA ATCACGAAAG ATGCACTAA ACATATGATA
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 1561 TACTTTAAAAA AGATATAAT GTTAATAGAT TATTAACCTAG TTATTCCTAAC GAAATATATA
 1621 AACATTAGA CATTACATTA TGTAAATATC TTATACGACG TGCAAGCAGAC ATAAACATTA
 1681 TAGATAAGAA CAATCGTACA CCGTTGTTT ATGCGGTAAA GAATAATGAT TATGATATGG
 1741 TTAAACTCCT ATTAAAAAAT GGCGCGAATG TAAATTTACA AGATAGTATA GGATATTCT
 1801 GTCTTCACAT CGCAGGTATA CATAATAGTA ACATAGAAAT AGTAGATGCA TTGATATCAT
 1861 ACAAAACCAGA TTAAACTCC CGCGATTGGG TAGGTAGAAC ACCGCTACAT ATCTCTGTGA
 1921 TAGAATCTAA CTTTGAAGCT GTGAAATTAT TATTAAGTC AGGTGCTATAT GTAGGTTG
 1981 AAGACAAATG TAAGCATTTT CCTATACACC ATTCTGTAAT GAAATTAGAT CACTTAATAT
 2041 CAGGATTGTT ATTAAAAAAT GGAGCAATC CAAATACAAAT TAACGGCAAT GGAAAAAACAT
 2101 TATTAAGCAT TGTGTAACA TCTAATATA CACTACTGGT AGAACAGCTG CTGTTATATG
 2161 GAGCAGAAAGT TAATAATGGT GGTTATGATG TTCCAGCTCC TATTATATCC GCTGTCAGTG
 2221 TTAACAAATT TAATGTTTAAAGTACAG TACATAATGG TGCGAATATA AATGTATCCA
 2281 CGGAAGATGG TAGAACGTCT TTACATACAG CTATGTTTTG GAATAACGCT AAAATAATAG
 2341 ATGAGTTGCT TAACTATGGG AGTGTACATA ACAGCGTAGA TACTTATGGT AGAAACTCCGT
 2401 TATCTGTGTT TCGTAGCTTA AGTTATGATA TCGCTACTAA ACTAAATATCA CGTATCATT
 2461 TAACAGATGT CTATCGTGAAC GCAACAGTAA ATATCAGCGG ATTATATAATT AATTAAAAAA
 2521 CTATAGAAAA TAATGATATA TTCAAATTAAT TAAAGATGA TTGTATTTAA GAGATAAAC
 2581 TACTTTAAAG TATAACCCCTT AATAAATTC ATTCTATCTGA CATATTTATA CGATATAATA
 2641 CTGATATATG TTATTAACG AGATTTATTC AACATCCAAA GATAATAGAA CTAGACAAAA
 2701 AACTCTACGC TTATAAATCT ATAGTCACG AGAGAAAAAT CAAAGCTACT TACAGGTATT
 2761 ATCAAATAAA AAAAGTATTA ACTGTACTAC CTTTTTCAGG ATATTTCTCT ATATTGCCGT
 2821 TTGATGTGTT AGTATATATA CTTGAATTCA TCTATGATAA TAATATGTT GTACTTATGA

FIG. 14A

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2941 AATGGCAGGA ATTTGTGTA AACTAAGCCA CATACTTGCC AATGAAAAAA ATAGTAGAAA
 3001 GGATACTATT TTAATGGGAT TAGATGTTAA GGTTCTTGG GATTATAGTA ACTGGGCATC
 3061 TGTTAACTTT TACGACGTTA GGTTAGATAC TGATGTTACA GATTATAATA ATGTTACAAT
 3121 AAAATACATG ACAGGATGTG ATATTTTCC TCATAACT CTTGGAATAG CAAATATGGA
 3181 TCAATGTGAT AGATTTGAAA ATTCAAAAA GCAATAACT GATCAAGATT TACAGACTAT
 3241 TTCTATAGTC TGAAAGAAG AGATGTGTT TCCTCAGAGT AACGCCTCTA AACAGTGGG
 3301 AGCGAAAGGA TCGCCTGAG TTATGAACT GGAGGTATCT GATGAACTTA GAGCCCTAAG
 3361 AAATGTTCTG CTGAATGCGG TACCCCTGTC GAAGGACGTTG TTTGGTGATA TCACAGTAGA
 3421 TAATCCGTGG ATTCCTCACA TAACAGTAGG ATATGTTAAG GAGGACGATG TCGAAAACAA
 3481 GAAACGCCTA ATGGAGTGC TGTCAGTT TAGGGGGCAA GAAATACAAG TTCTAGGATG
 3541 GTATTAATAA GATCTAAGT ATTTGGTATA ATTTTATAAA TAGTATAATT ATAACAAATA
 3601 ATAAATAACA TGATAACGGT TTTTATTAGA ATAAAATAGA GATAATATCA TAATGATATA
 3661 TAATACTTCA TTACCAAGAAA TGAGTAATGG AAGACTTATA ATGAACTGC ATAAAGCTAT
 3721 AAGGTATAGA GATATAAATT TAGTAAGGT TATACTTAAA AATGCAAAT ACAATAACGT
 3781 AAATATACTA TCAACGTTCT TGATTTAGC CGTAAGTATT TCTGATATAG AAATGGTAAA
 3841 ATTATTACTA GAACACGGTG CCGATATTAA AAAATGTTAA ATACCTCCTC TTCATAAAGC
 3901 TGCTAGTTA GATAATACAG AAATTGCTAA ACTACTAATA GATTCTGGCG CTGACATAGA
 3961 ACAGATACAT TCTGGAAATA GTCCGTTATA TATTCTGTA TATAGAAACA ATAAGTCATT
 4021 AACTAGATAT TTATTAAGG AAGGTGTTAA TTGTAATAGA TTCTTTCTAA ATTATTACGA
 4081 TGACTGTAT GATAAGATAT CTGATGATAT GTATAAATAA TTTATAGATT TTAATATTGA
 4141 TCTTAATATA CAAACTAGAA ATTTGAAAC TCCGTTACAT TAGCTATAAA AGTATAAGAA
 4201 TATAGATTTA ATTAGGGATAT GTTGTAGATAA TAGTATTAAA ATAGATAAAA GTTATTTT
 4261 GCATAAACAG TATCTCATAA AGGCACTTAA AAATAATTGT AGTTACGATA TAATAGCGTT
 4321 ACTTATAAT CACGGAGTGC CTATAAACGA ACAAGATGAT TTAGGTAAAA CCCCATTACA
 4381 TCATTGGTA ATTAATAGAA GAAAAGATGT AACAGCACTT CTGTTAAATC TAGGAGCTGA
 4441 TATAAACGTA ATAGATGACT GTATGGGAG TCCCTTACAT TAGCTGTTT CACGTAACGA
 4501 TATCGAAACA ACAAAAGACAC TTTAGAAAAG AGGATCTAA GTTAATGTGG TTAATATCA
 4561 TATAGATACC GTTCTAAATA TAGCTGTGTC ATCTAAAAAC AAAACTATAG TAAACTTATT
 4621 ACTGAAGTAC GGTACTGATA CAAAGTTGGT AGGATTAGAT AAACATGTTA TTCACATAGC
 4681 TATAGAAATG AAAGATATTA ATATACTGAA TGCGATCTTA TTATATGGTT GCTATGTTAA
 4741 CGTCTATAAT CATAAAGGTT TCACTCCCTC ATACATGGCA GTTGTCTCTA TGAAAACAGA
 4801 ATTTGTTAAA CTCTTACTTG ACCACGGTGC TTACGTAAT GCTAAAGCTA AGTTATCTGG
 4861 AAATACTCCT TTACATAAAG CTATGTTATC TAATGTTTT AATAATATAA AATTACTTTT
 4921 ATCTTATAAC GCCGACTATA ATTCTCTAA TAATCACGGT AATACGCCCTC TAACCTGTGT
 4981 TAGCTTTTA GATGACAAGA TAGCTATTAT GATAATATCT AAAATGATGT TAGAAATATC
 5041 TAAAAATCCT GAAATAGCTA ATTCAAGAAGG TTTTATAGTA AACATGGAAC ATATAAACAG
 5101 TAATAAAAGA CTACTATCTA TAAAAGAATC ATGCGAAAAAA GAACTAGATG TTATAACACA
 5161 TATAAAGTTA AATTCTATAT ATTCTTTAA TATCTTCTT GACAATAACA TAGATCTTAT
 5221 GGTAAGTTC GTAACTAATC CTAGAGTTAA TAAGATACCT GCATGTATAC GTATATATAG
 5281 GGAATTAAATA CGGAAAAATA AATCATTAGC TTTTCATAGA CATCAGCTAA TAGTTAAAGC
 5341 TGAAAAGAG AGTAAAGAATC TAGGAATAAT AGGTAGGTTA CCTATAGATA TCAAAACATAT
 5401 AATAATGGAA CTATTAAGTA ATAATGATT ACATCTGTT ATCACCAGCT GTTGTACCC
 5461 AGTAGTATAA AGTGATTTA TTCAATTAGC AAGATAAAACA TTAAATTTGT TAACAGATAT
 5521 GAGTTATGAG TATTTAACTA AAGTTACTTT AGGTACAAT AAAATATTAT GTAATATAAT
 5581 AGAAAATTAT CTTGAGTCTT CATTCCATC ACCGTCTAA TTTATTATTA AAACCTTATT
 5641 ATATAAGGCT GTTGAGTTA GAAATGTTAA TGCTGTAAAA AAAATATTAC AGAATGATAT
 5701 TGAATATGTT AAAGTAGATA GTCATGGTGT CTCGCCCTTA CATATTATAG CTATGCCCTC
 5761 AAATTTTCT CTCATAGACG CTGACATGTA TTCAGAATT AATGAAATTA GTAATAGACT

FIG. 14B

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5821 TCAAAAATCT AAAGATAGTA ACGAATTCA ACGAGTTAGT CTACTAAGGA CAATTATAGA
 5881 ATATGGTAAT GATAGTGATA TTAATAAGTG TCTAACATTA GTAAAAAACGG ATATACAGAG
 5941 TAACGAAGAG ATAGATATTAGATCTTTT GATAAATAAA GGAATAGATA TAAATATTAAG
 6001 AGACGATTAGGAAACACAG CTTTGCATTA CTCGTGTGAT TATGCTAAGG GATCAAAGAT
 6061 AGCTAAAAAG TTACTAGATT GTGGAGCAGA TCCTAACATA GTTAATGATT TAGGTGTTAC
 6121 ACCACTAGCG TGTGCCGTTA ATACTTGCAA CGAGATACTA GTAGATATTCT GTTTAAATAA
 6181 TGATGCGAAT CCTGATTCACTTCCTCATA TTTTTAGGT ACTAATGTGT TACATACAGC
 6241 CGTAGGTACCGTAATATAG ATATTGTAG ATCTTTACTT ACGGCTGGTG CCAATCCTAA
 6301 TGTAGGAGAT AAATCTGGAG TTACTCCTT GCACGTTGCT GCAGCTGATA AAGACAGTTA
 6361 TCTGTTAATG GAGATGCTAC TAGATAGCGG GGCAGATCCA AATATAAAT GCGCAAACGG
 6421 TTTTACTCCT TTGTTAATG CAGTATATGA TCATAACCGT AAAAAAGTTAT TTTTCTTA
 6481 CGGGGCTGAT ATCAATATTA CTGACTCTTA CGGAAATACT CCTCTTACTT ATATGACTAA
 6541 TTTTGATAAT AAATATGTAA ATTCAATAAT TATCTTACAA ATATATCTAC TTAAAAAAAAGA
 6601 ATATAACGAT GAAAGATTGT TTCCACCTGG TATGATAAAA AATTTAAACT TTATAGAATC
 6661 AAACGATAGT CTTAAAGTTA TAGCTAAAAA GTGTAATTG TTAATACGCT ATAAGAAAAA
 6721 TAAAGACATA GATGCAGATA ACGTATTATT GGAGCTTTA GAGGAAGAGG AAGAAGATGA
 6781 AATAGACAGA TGGCATACTA CATGAAAAAT ATCTTAAATA GAAATTAAAT CATTGAAATA
 6841 TTAACTTACA AGATGATCGA GGTCACTTAT TATACTCTT AATAATGGGT ACAAAGAGTA
 6901 TTCATACGTT AGTTAAATCT AACGATGTTA TACGTGTTCG TGAATTAAATA AAGGGATGATA
 6961 GATGTTTGAT AAATAAAAAGA AATAGAAGAA ATCAGTCACC TGATATATA GCTATATACA
 7021 AAGGACTTTA TGAAATGACT GAAATGTTAT TGCTAAATAA TGCAAGTCTA GATACTAAAA
 7081 TACCTTCTTT AATTATAGCA GCTAAAAATA ATGACTTACCT ATGATAAAA TTATTGATAC
 7141 AATACGGGGC AAAATTAAAT GATATTATT TAAGGGACAC AGCATTAAATG ATAGCTCTCA
 7201 GAAATGGTTA CCTAGATATA GCTGAATATT TACTTTCTT AGGAGCAGAA TTTGTTAAAT
 7261 ACAGACATAA GGTAAATAT AATATCTAT CAAAGATGC GTATGAATTA CTTTTAGAT
 7321 TTAATTATGA CGTTAATATA ATAGATTGAG A

FIG. 14C

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1 AAAAAGGATCCGGGTTAATTAAATTAGTCATCAGGCAGGGCGAGAACGAGACTATCTGCTC
 61 GTTAATTAAATTAGAGCTTCTTATTCTACTTAAAGTAAAATAAAACAAAGGTTCTT
 121 TTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAAATTATTCATTATCGCGATA
 181 TCCGTTAAGTTGTATCGTAATGAGCACTGAAAGCATGATCAGGGACGTGGAGCTGGCCG
 241 AGGAGGCCTCCAGAAGACAGGGGGCCCGAGGGCTCCAGGCGGTGCTTGTCTCA
 301 GCCTCTTCTCCTCTGATCGTGGCAGGCACCGCTCTCTGCCTGCTGCACTTG
 361 GAGTGAATGGCCCCCAGAGGGAAAGGTCCCCCAGGGACCTCTCTAATCAGCCCTCTGG
 421 CCCAGGCAGTCAGATCATCTTCAGAACCCGAGTGACAAGGCTGTAGCCCCATGTTGAG
 481 CAAACCCCTAAGCTGAGGGGAGCTCCAGTGGCTGAACCGCCGGGCAATGCCCTCTGG
 541 CCAATGGCGTGGAGCTGAGAGATAACCAAGCTGGTGGTGCATCAGAGGGCTGTACCTCA
 601 TCTACTCCCAGGTCTCTCAAGGGCCAAGGCTGCCCTCCACCCATGTGCTCTCACCC
 661 ACACCACTAGCCGCATCGCCGCTCTCTAACAGACCAAGGTCAACCTCCCTCTGCCATCA
 721 AGAGCCCCCTGCCAGAGGGAGACCCAGAGGGGGCTGAGGCAAGGCCCTGGTATGAGGCCA
 781 TCTATCTGGGAGGGGTCTTCAGCTGGAGAAGGGTGACCGACTCAGCGCTGAGATCAATC
 841 GGCCCGACTATCTCGACTTGGCGAGTCTGGCAGGTCTACTTGGGATCATTGCCCTGT
 901 GATTTTATTCTAGAATCGATCCGGGTTTTATGACTAGTTAATCACGGCCGTTATAA
 961 AGATC

FIG. 15

1 GATCTCAAGCCGTCTTCTGGTGTAAATAAAATTAAATTACTCGAGCCCAGCTTGA
 61 TTCTTATTCTATACTTAAAGTAAAATAAAACAAAGGTTCTGAGGGTTGTGTTAA
 121 ATTGAAAGCGAGAAATAATCATAAAATTATTCATTATCGCGATATCCGTTAAGTTGTAT
 181 CGTAATGAGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGAGGGAGGGCGCTCCCAA
 241 GAAGACAGGGGGGCCCCAGGGCTCCAGGGCGGTGCTTCTCAGCCTCTCTCCCT
 301 GATCGTGGCAGGCACCCACGCTCTGCTGCACTTGGAGTGATCGGCCCCA
 361 GAGGGAAAGAGTCCCCCAGGGACCTCTCTTAATCAGCCCTCTGGCCCAAGGCAGTCAGATC
 421 ATCTTCTCGAACCCCGAGTGACAAGCCTGAGCCATGTTGAGCAAACCCCTCAAGCTGA
 481 GGGGCAGCTCAGTGGCTGAACCGCCGGCCAATGCCCTCCTGGCCAATGGCGTGGAGCT
 541 GAGAGATAACCAAGCTGGTGGTGCATCAGAGGGCTGTACCTCATCTACTCCAGGTCT
 601 CTTCAAGGGCCAAGGCTGCCCTCCACCCATGTGCTCCTCACCCACACCATCAGCCGCAT
 661 CGCGTCTCCTACCAAGACCAAGGTCAACCTCTCTGCCATCAAGAGGCCCTGCCAGAG
 721 GGAGACCCCAAGAGGGGGCTGAGGCCAAGCCCTGGTATGAGCCCATCTATCTGGGAGGGGT
 781 CTTCCAGCTGGAGAAGGGTGACCGACTCAGCGCTGAGATCAATCGGCCGACTATCTCGA
 841 CTTTGGCGAGTCTGGCAGGTCTACTTGGGATCATTGCCCTGTGATTTTATTGGGAGA
 901 TCTAATTAAATTATAACTTATTTGAATATACTTTA

FIG. 16

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1 GATTAAAGAAAGTTACTCTGAGACACAAAAGAGGTAGCTGAAGTGGTACTCTCAAAGGTA
 61 CCCCCGGGTTAATTAAATTAGTCATCAGCAGGGCAGAGACAGACTATCTGCTCGTTAAT
 121 TAATTAGGTGACGGATCCCCGGGTTCTTATTCTATACTTAAAGTGAAAATAATAC
 181 AAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTCATT
 241 TCGCGATATCCGTTAAGTTGATCGTAATGGAGGAGCCGAGTCAGATCCTAGCGTCGA
 301 GCCCCCTCTGAGTCAGGAAACATTTCAGACCTATGGAAACTACTTCCTGAAAACACGT
 361 TCTGTCCCCCTGCCGTCCCAAGCAATTGGATGATTGATGCTGCCCCGGACGATATTGA
 421 ACAATGGTTCACTGAAAGACCCAGGTCCAGATGAAGCTCCAGAATGCCAGAGGCTGCTCC
 481 CGCGTGGCCCTGGACCAGCAGCTCTACACCGCGGCCCTGCACCAGCCCCCTCCTG
 541 GCCCCGTGTCATCTTCTGTCCTTCCCAGAAAACCTACCAGGGCAGCTACGGTTCCGTCT
 601 GGGCTTCTGCAATTCTGGGACAGCCAAGTCTGTGACTTGACCGTACTCCCCGCCCCCTCAA
 661 CAAGATGTTTGCCAACCTGGCCAAAGACCTGCCCCCTGTCAGCAGCTGGGTTGATTCCACACC
 721 CCCGCCCGCACCCCGCTCCGCGCCATGGCATCTACAAGCAGTCACAGCACATGACGGA
 781 GGTTGTGAGGCGCTGCCCAACCATGAGCGCTGCTCAGATAGCGATGGTCTGGCCCTCC
 841 TCAGCATCTTATCCGAGTGGAAAGGAAATTGCGTGTGGAGTATTGGATGACAGAAACAC
 901 TTTCGACATAGTGTGTTGGTGCCTATGAGCCGCTGAGGGTGGCTGTACTGTACCAAC
 961 CATCCACTACAACATACATGTAAACAGTTCTGCATGGCGGCATGAACCGGAGGGCCAT
 1021 CCTCACCATCATCACACTGGAAAGACTCCAGTGGTAATCTACTGGGACGGAAACAGCTTGA
 1081 GGTGCGTGTGCTGTGCTCTGGGAGAGACCGGGCGCACAGAGGAAGAGAATCTCCGCAA
 1141 GAAAGGGGAGCCTCACCAACAGAGCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCAAACAA
 1201 CACCAGCTCCTCTCCCCAGCCAAGAAGAAACCAACTGGATGGAGAATATTCACCCCTCA
 1261 GATCCGTTGGCGTGAGCGCTTGAGATGTTCCGAGAGCTGAATGAGGCCCTGGAACACTCAA
 1321 GGATGCCCAGGCTGGGAAGGAGCCAGGGGGAGCAGGGCTCACTCCAGGCCACCTGAAGTC
 1381 CAAAAAGGGTCAGTCTACCTCCGCCATAAAAAAAACTCATGTTCAAGACAGAAGGGCCTGA
 1441 CTCAGACTGAACCGCTTTTATCCGGGCTCGAGTCTAGAATCGATCCGGGTTTATG
 1501 ACTAGTTAATCA

FIG. 17

1 GGTACGTGACTAATTAGCTATAAAAGGATCTTAATTAAATTAGTCATCAGGCAGGGCGAG
 61 AACGAGACTATCTGCTCGTTAATTAAATTAGGTGACGGATCCCCGGGTTCTTATTCTA
 121 TACTTAAAGTGAAAATAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCAG
 181 AAATAATCATAAATTATTCATTATCGCGATATCGTTAAGTTGATCGTAATGGAGGA
 241 GCCGCAGTCAGATCCTAGCGTCAGCCCCCTCTGAGTCAGGAAACATTTCAGACCTATG
 301 GAAACTACTCCTGAAAACAACGTTCTGTCCTTGCCTCCCAAGCAATGGATGATT
 361 GATGCTGCCCCGGACGATATTGAAACATGGTCACTGAAGACCCAGGTCCAGATGAAGC
 421 TCCCAGAATGCCAGAGGCTGCTCCCCCGCTGGCCCTGACAGCAGCTCCTACACCGGC
 481 GGCCCCTGCACCAGCCCCCTCTGGCCCTGTCATCTTCTGCCCCCTCCAGAAAACCTA
 541 CCAGGGCAGCTACGGTTCCGTCTGGGTTCTTGCAATTCTGGACAGCCAAGTCTGTAC
 601 TTGACGTACTCCCCGCCCCCTCAACAAGATGTTTGCAACTGGCAAGACCTGCCCTGT
 661 GCAGCTGGGTTGATTCCACACCCCGCCGACCCCGCTCCGCGCCATGGCCATCTA
 721 CAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCCTGCCCCCACCATGAGCGCTGCTC
 781 AGATAGCGATGGTCTGGCCCTCCTCAGCATCTTATCCGAGTGGAAAGGAAATTGCGTGT
 841 GGAGTATTGGATGACAGAAACACTTTGACATAGTGTGGTGTGCCCCATGAGCCGCC
 901 TGAGGTTGGCTCTGACTGTACCAACATCAACTACATGTAAACAGTTCTGCAT
 961 GGGCGGCATGAACCCGGAGGCCATCTCACCATCATCACACTGGAAAGACTCCAGTGGTAA
 1021 TCTACTGGGACGGAACAGCTTGAGGTGCGTGTGGCTGCTGGAGAGACCGGGCG
 1081 CACAGAGGAAGAGAAATCTCCGCAAGAAAGGGGAGGCTCACCAAGCAGCTGCCAGGGAG
 1141 CACTAAGCGAGCACTGCCAACACACCCAGCTCCTCTCCCCAGCCAAGAAGAAACCACT
 1201 GGATGGAGAATATTTCACCCCTCAGATCCGTTGGGCGTGAAGCCTGGAGATGTTCCGAGA
 1261 GCTGAATGAGGCCTGGAACTCAAGGATGCCAGGGAGCCAGGGGGAGCAG
 1321 GGCTCACTCCAGCCACCTGAAGTCCAAAAAGGGTCAAGTCTACCTCCGCCATAAAAAAAACT
 1381 CATGTTCAAGACAGAAGGGCCTGACTCAGACTGAACCGCTGTTTATCCGGGCTCGAGTC
 1441 TAGAATCGATCCGGGTTTATGACTAGTTAATCACGGCCG

FIG. 18**SUBSTITUTE SHEET (RULE 26)**

1 AAAAAGGATCCGGGTTAATTAAATTAGTCATCAGGCAGGGCGAGAACGAGACTATCTGCTC
 61 GTTAATTAAATTAGAGCTCTTATTCTATACTTAAAGTAAAATAACAAAGGTT
 121 TTGAGGGTTGTGTTAAATTGAAAGCGAGAAAATAATCATAAAATTATTCATTATCGCGATA
 181 TCCGTTAACGTTGAGGCTGTAATGTCCTTGAGCAGAGGAGTCTGCACTGCAAGCCTGAGG
 241 AAGCCCTGAGGCCAACAAAGAGGCCCTGGGCCTGGTGTGTGCAGGCTGCCACCTCCT
 301 CCTCCTCTCCTCTGGCTGGCACCCCTGGAGGGAGGTGCCACTGCTGGGTCAACAGATC
 361 CTCCCCAGAGTCCTCAGGGAGCCTCCGCCTTCCACTACCATCAACCTCACTGACAGA
 421 GGCAACCCAGTGAGGGTTCCAGCAGCCGTGAAGAGGGAGGGGCCAAGCACCTTGTATCC
 481 TGGAGTCCTGTTCCAGCAGTAATCACTAAGAAGGTGGCTGATTGGTTGGTTTCTGC
 541 TCCTCAAATATCGAGCCAGGGAGCCAGTCACAAAGGCAGAAATGCTGGAGAGTGTCA
 601 AAAATTACAAGCACTGTTTCTGAGATCTTCGGCAAAGCCTCTGAGTCCTGCAGCTGG
 661 TCTTGGCATTGACGTGAAGGAAGCAGACCCCCACCGGCCACTCCTATGTCCTTGTCACCT
 721 GCCTAGGTCTCTCTATGATGGCCTGCTGGGTGATAATCAGATCATGCCAAGACAGGCT
 781 TCCTGATAATTGTCCTGGTCAATTGCAATGGAGGGCGGCCATGCTCTGAGGAGGAAA
 841 TCTGGGAGGGAGCTGAGTGTGATGGAGGTGTATGATGGGAGGGAGCACAGTGCCTATGGGG
 901 AGCCCAGGAAGCTGCTCACCCAAAGATTGGTGCAGGAAAAGTACCTGGAGTACGGCAGGT
 961 GCCGGACAGTGTACCCGCACGCTATGAGTTCTGTGGGTCCAAGGGCCCTGCTGAAAC
 1021 CAGCTATGTGATTTTATTCTAGAATCGATCCC GGTTTTATGACTAGTTAATCACGGC
 1081 CGCTTATAAAGATC

FIG. 19

1 ATAAATCACTTTTATACTAATATTAATTAAAGCTTGGTACCCCTCGAAGCTTCTT
 61 ATTCTATACTAAAAAGTAAAATAACAAAGGTTCTTGAGGGTTGTGTTAAATTGAA
 121 AGCGAGAAAATAATCATAAAATTATTCATTATCGCGATATCCGTTAACGTTGATCGTAAT
 181 GTCTCTTGAGCAGAGGAGTCTGCACTGCAAGCCTGAGGAAGGCCCTGAGGCCCCAACAAAGA
 241 GGCCCTGGGCCTGGTGTGTCAGGCTGCCACCTCCTCTCCTCTGGTCCTGG
 301 CACCCCTGGAGGGAGGTGCCACTGCTGGGTCAACAGATCCTCCCCAGAGTCCTCAGGGAGC
 361 CTCCGCCCTTCCCACCTACCATCAACTCACTGACAGAGGCAACCCAGTGAGGGTTCCAG
 421 CAGCCGTGAAGAGGGAGGGCCAAGCACCTCTGTATCTGGAGTCCTTGTGAGCAGT
 481 AATCACTAAGAAGGTGGCTGTTGGTTTCTGCTCCTCAAATATCGAGCCAGGGA
 541 GCCAGTCACAAGGGCAGAAATGCTGGAGAGTGTCAAAATTACAAGCACTGTTTCC
 601 TGAGATCTCGGCAAAGCCTCTGAGTCCTTGAGCTGGTCTTGGCATTGACGTGAAGGA
 661 AGCAGACCCCCACCGGCCACTCTATGTCCTTGTCACCTGCCTAGGTCTCTCTATGATGG
 721 CCTGCTGGGTGATAATCAGATCATGCCAAGACAGGCTCCTGATAATTGTCCTGGTCAT
 781 GATTGCAATGGAGGGCGGCCATGCTCTGAGGAGGAAATCTGGGAGGGAGCTGAGTGTGAT
 841 GGAGGTGATGATGGGAGGGAGCACAGTGCCTATGGGGAGGCCAGGAAGCTGCTCACCCA
 901 AGATTGGTGCAGGAAAAGTACCTGGAGTACGGCAGGTGCCGGACAGTGATCCGCACGC
 961 TATGAGTCTGTGGGTCCAAGGGCCCTGCTGAAACCAGCTATGTGATTTTATTCTA
 1021 GAACTAGTGGATCCCCGGGTAGCTAGCTAATTCTTACGTATTATATGTAATA
 1081 AACG

FIG.20

SUBSTITUTE SHEET (RULE 26)

FIG. 21A

TGGAATTGAAATAAGAGGAGTTAGTATTACCTGAAATTAAATTGATTAGAAATTTCATGAACATTATAATGATAAAAATTCTCAGAAAGATAAAAGAT 1575
 AATAAAGGAAAGGTGTCGATCACAACTTGTGATCAAGGTAAATTGATAACTCGATAATATGTTTGTAAATAAGAAATATTGTAAC 1680
 AGATTAAAGATGAAATAATTGATAATCTTAATCTTCAGGTAAAGGTAAATTGATAACTTCATGGATTTTGCTTCAAAATATCATTAGAAACTATT 1785
 AGATGTATGAAAGGATATGAAACCTAACCCAAATTCTGTCTTTATGTAGCTAATTGTTATAAAGGTGAACATAAAGATAGATGTGATGAAGGTTCTAGTCCAATG 1890
 GAAATTCTACAAATTATGAAAGATTATGGATTCTTACCGAGAACTCAAAATTATCCATAACTATGTGAAAGTTGGAGAACATACTATGTGAAAGGTTGAGAATCAC 1995
 TGATGAACTCATGGGATAATGGAAATAACAAAATGAAACCTAATAGTTAGATGGTAAGGGATAACTGCATAATGAAAGTGAAGGTTATGATGAT 2100
 AATATGGATGCAATTGTTAAAGGTCAATAATGAAATAAGGTCAAGTTATTGGCATAATTAAAGCTGAAAAATGTTATGGGATATGAAATTAGTGGAA 2205
 AAGAAAAGTACAGAAACTTATGTTGGTGTATCACAGCTGATCATGCAAGTTAATTTGGTTAATTATGTAAGTGGAGAAAAAAATCCTATTGG 2310
 ATTGTAAGAAACAGTTGGGTCATATTGGGAGATGAAGGTTATTAAAGTAGATATGTTAAGCTCATTTAACTTTAATTCACTGTTGTT 2415
 ATATTCAATGTTGATTACCTATGAAATAAAACAACTAAAAAGAATCAAATATGATTATTAAAGGCTCTCCAGAAATTTATCATAAACCTTTAC 2520
 TTAAAGAAATTAAATGTTGAAAGGAAAGATAATTGAAACAAACAAACAAATTAGGTAAACACTATTATTCGGTCAGAAGCTCATGTTTACATATAAAACAT 2625
 GCAGGATCAGGACAAAGTGGAAAGGAAACTGCAATTAGAAATCTGCATTAGAAATCTGCAGGAAACTTCAGAACGTTGTTCATGTTTACATATAAAACAT 2730
 ATAAAGGATGGCAAAATAGAATGGGTATGCGTAATATAAGATAACAAAGATTGAAATAGAAACATTCTGTGAAAGATCTCATGCAATTAAATCCAGAGAAT 2835
 ATGAAAATGTTGAAATTATGTAATGTGAAACTGGGAAACATGCGGAGAAAACATGCGGAGAAAATGCGGAGAAAACATGCGGAGAAAATGTTATGTT 2940
 TTCTGTTATGTTAAATAACAAAAAAACATGCGGAGAAAATGCGGAGAAAATGCGGAGAAAATGCGGAGAAAATGCGGAGAAAATGCGGAGAAAATGTT 2981
 F C Y V

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1	GAAGCAATAG	CTTGTATGCT	TTTATTGTA	TTAACTAGTC	ATAAAAATCG	GGATCCTTCT
61	TTATTCTATA	CTTAAAAAGT	GAAAATAAAT	ACAAAGGTTT	TTGAGGGTTG	TGTTAAATTG
121	AAAGCGAGAA	ATAATCATAA	ATTATTCAT	TATCGCGATA	TCCGTTAAGT	TTGTATCGTA
181	ATGGAGTCTC	CCTCGGCCCC	TCCCCACAGA	TGGTGCATCC	CCTGGCAGAG	GCTCCTGCTC
241	ACAGCCTCAC	TTCTAACCTT	CTGGAACCCG	CCCACCACTG	CCAAGCTCAC	TATTGAATCC
301	ACGCCGTTCA	ATGTCGCAGA	GGGGAAGGAG	GTGCTTCTAC	TTGTCCACAA	TCTGCCAG
361	CATCTTTTG	GCTACAGCTG	GTACAAAGGT	GAAAGAGTGG	ATGGCAACCG	TCAAATTATA
421	GGATATGTAA	TAGGAACTCA	ACAAGCTACC	CCAGGCCCG	CATACAGTGG	TCGAGAGATA
481	ATATAACCCA	ATGCATCCCT	GCTGATCCAG	AACATCATCC	AGAATGACAC	AGGATTCTAC
541	ACCCCTACACG	TCATAAAAGTC	AGATCTTGTG	AATGAAGAAG	CAACTGGCCA	GTTCCGGGTA
601	TACCCGGAGC	TGCCCAAGCC	CTCCATCTCC	AGCAACAAC	CAAACCCGT	GGAGGAACAG
661	GATGCTGTGG	CCTTCACCTG	TGAACCTGAG	ACTCAGGACG	CAACCTACCT	GTGGTGGGTA
721	AAACAATCAGA	GCCTCCCGGT	CAGTCCCAAGG	CTGCACTGTG	CCAATGGCAA	CAGGACCCCTC
781	ACTCTATTCA	ATGTCACAAG	AAATGACACA	GCAAGCTACA	AATGTGAAAC	CCAGAACCCA
841	GTGAGTGCCA	GGCGCAGTGA	TTCAGTCATC	CTGAATGTCC	TCTATGGCCC	GGATGCCCC
901	ACCATTCCCC	CTCTAAACAC	ATCTTACAC	TCAGGGAAA	ATCTGAACCT	CTCCTGCCAC
961	GCAGCCTCTA	ACCCACCTGC	ACAGTACTCT	TGGTTTGTCA	ATGGGACTTT	CCAGCAATCC
1021	ACCCAAGAGC	TCTTTATCCC	CAACATCACT	GTGAATAATA	GTGGATCCTA	TACGTGCCAA
1081	GCCCCATAACT	CAGACACTGG	CCTCAATAGG	ACCACAGTCA	CGACGATCAC	AGTCTATGCA
1141	GAGGCCACCA	AACCCTTCAT	CACCAAGAAC	AACTCCAACC	CCGTGGAGGA	TGAGGATGCT
1201	GTAGCCTTAA	CCTGTGAACC	TGAGATTCTAG	AACACAACCT	ACCTGTGGTG	GGTAAATAAT
1261	CAGAGCCTCC	CGGTCAGTCC	CAGGCTGAG	CTGTCCTAATG	ACAACAGGAC	CCTCACTCTA
1321	CTCACTGTCA	CAAGGAATGA	TGTAGGACCC	TATGAGTGTG	GAATCCAGAA	CGAATTAAAGT
1381	GTTGACCCACA	GCGACCCAGT	CATCCTGAAT	GTCTCTATG	GCCCCAGACGA	CCCCACCAT
1441	TCCCCCTCAT	ACACCTATT	CCGTCAGGG	GTGAACCTCA	GCCTCTCTG	CCATGCAGCC
1501	TCTAACCCAC	CTGCACAGTA	TTCTTGGCTG	ATTGATGGGA	ACATCCAGCA	ACACACACAA
1561	GAGCTCTTA	TCTCCAACAT	CACTGAGAAG	AACAGCGGAC	TCTATACCTG	CCAGGCCAAT
1621	AACTCAGCCA	GTGGCCACAG	CAGGACTACA	GTCAAGACAA	TCACAGTCTC	TGCGGAGCTG
1681	CCCAAGCCCT	CCATCTCCAG	CAACAACCTC	AAACCCGTGG	AGGACAAGGA	TGCTGTGGCC
1741	TTCACCTGTG	AACTCTGAGGC	TCAGAACACA	ACCTACCTGT	GGTGGGTAAG	TGGTCAGAGC
1801	CTCCCAGTCA	GTCCCAGGCT	GCAGCTGTCC	AATGGCAACA	GGACCCCTCAC	TCTATTCAAT
1861	GTCACAAGAA	ATGACGCAAG	AGCCTATGTA	TGTGGATCC	AGAACTCAGT	GAGTGCAAAC
1921	CGCAGTGACC	CAGTCACCCCT	GGATGTCTC	TATGGGCCGG	ACACCCCCAT	CATTTCCCC
1981	CCAGACTCGT	CTTACCTTTC	GGGAGCGAAC	CTCAACCTCT	CCTGCCACTC	GGCCTCTAAC
2041	CCATCCCCGC	AGTATTCTTG	CGCTATCAAT	GGGATACCAC	AGCAACACAC	ACAAGTTCTC
2101	TTTATCGCCA	AAATCACGCC	AAATAATAAC	GGGACCTATG	CCTGTTTGT	CTCTAACTTG
2161	GCTACTGGCC	GCAATAATT	CATAGTCAG	AGCATCACAG	TCTCTGCATC	TGGAACCTCT
2221	CCTGGTCTCT	CAGCTGGGGC	CACTGTCGGC	ATCATGATTG	GAGTGCTGGT	TGGGGTTGCT
2281	CTGATATAGT	TTTTATCTCG	AGGAATTCCT	GCAGCCGGG	GTGACCTAAT	TAATTAAGCT
2341	ACAAATAGTT	TCGTTTCACT	CTTGTCTAAT	AACTAATTAA	TTAACCGGGT	TTTTATAGCT
2401	AATTAGTCAA	ATGTGAGTTA	ATATTAGTAT	ACTA		

FIG.22

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1 TAAAAAATAAA TCACTTTTA TACTAATATT TAATTAAATTA AGCTTGGTAC CCTCGAAGCT
 61 TCTTTATTCT ATACTAAAA AGTGAATAA AATACAAAGG TTCTTGAGGG TTGTGTTAAA
 121 TTGAAAGCGA GAAATAATCA TAAATTATT CATTATCGCG ATATCGTTA AGTTTGTATC
 181 GTAATGGAGT CTCCCTCGGC CCCTCCCCAC AGATGGTGC A TCCCCTGGCA GAGGCTCCG
 241 CTCACAGCCT CACITCTAAC CTTCTGGAAC CCGCCACCA CTGCCAAGCT CACTATTGAA
 301 TCCACGCCGT TCAATGTCGC AGAGGGGAAG GAGGTGCTTC TACTTGTCCA CAATCTGCC
 361 CAGCATTTT TTGGCTACAG CTGGTACAAA GGTGAAAGAG GGGATGGCAA CGTCAAATT
 421 ATAGGATATG TAATAGGAAC TCAACAAAGT ACCCCCAGGGC CGGCATACAG TGGTCGAGAG
 481 ATAATATACC CCAATGCATC CCTGCTGATC CAGAACATCA TCCAGAATGA CACAGGATT
 541 TACACCCCTAC ACGTCAATAA GTCAAGATCTT GTGAATGAAG AAGCAACTGG CCAGTTCCGG
 601 GTATACCCGG AGCTGCCAA GCCCTCCATC TCCAGCAACA ACTCCAAACC CGTGGAGGAC
 661 AAGGATGCTG TGGCCCTAC CTGTGAACCT GAGACTCAGG ACGCAACCTA CCTGTGGTGG
 721 GTAAACAAATC AGAGCCTCCC GGTCAGTCCC AGGCTGCAGC TGCCAATGG CAACAGGACC
 781 CTCACTCTAT TCAATGTCAC AAGAAATGAC ACAGCAAGCT ACAAATGTGA AACCCAGAAC
 841 CCAGTGAATG CCAGGCCAG TGATTCACTG ATCCTGAATG TCCCTATGG CCCGGATGCC
 901 CCCACCATTT CCCCTCTAAA CACATCTTAC AGATCAGGGG AAAATCTGAA CCTCTCTGC
 961 CACGCAGCCT CTAACCCACC TGACAGTAC TCTTGGTTT TCAATGGGAC TTTCCAGCAA
 1021 TCCACCCAAG AGCTCTTTAT CCCCAACATC ACTGTGAATA ATAGTGGATC CTATACGTGC
 1081 CAAGCCCAT AACTCAGACAC TGGCCTCAAT AGGACACAG TCACGACGAT CACAGTCTAT
 1141 GCAGAGCCAC CCAAAACCTT CATCACCAGC AACAACCTCA ACCCCGTGGA GGATGAGGAT
 1201 GCTGTAGCT TAACCTGTGA ACCTGAGATT CAGAACACAA CCTACCTGTG GTGGGTAAT
 1261 AATCAGAGCC TCCCAGGTCA TGCCAGGCTG CAGCTGTCCA ATGACAAACAG GACCCTCACT
 1321 CTACTCACTG TCACAAAGGA TGATGTAGGA CCCTATGAGT GTGGAATCCA GAACGAATT
 1381 AGTGTGACC ACAGCGACCC AGTCATCCTG AATGTCCTCT ATGGCCCAAGA CGACCCACC
 1441 ATTTCCCCCT CATAACACCTA TTACCGTCCA GGGGTGAACC TCAGCCTCTC CTGCCATGCA
 1501 GCCTCTAACCC CACCTGCACA GTATTCTTGG CTGATTGATG GGAACATCCA GCAACACACA
 1561 CAAGAGCTCT TTATCTCCAA CATCACTGAG AAGAACAGCG GACTCTATAC CTGCCAGGCC
 1621 AATAACTCAG CCAGTGGCCA CAGCAGGACT ACAGTCAAGA CAATCACAGT CTCTGCGGAG
 1681 CTGCCAACGC CCTCCATCTC CAGCAACAAAC TCCAAACCCG TGGAGGACAA GGATGCTGTG
 1741 GCCTTCACCT GTGAACCTGA GGCTCAGAAC ACAACACTACC TGTGGTGGGT AAATGGTCAG
 1801 AGCCTCCAG TCAGTCCCA GCTGCAGCTG TCCAATGGCA ACAGGACCCCT CACTCTATT
 1861 AATGTACAA GAAATGACGC AAGAGCCTAT GTATGTGGAA TCCAGAACTC AGTGAATGCA
 1921 AACCGCAGTG ACCCAAGTCAC CCTGGATGTC CTCTATGGGC CGGACACCCCC CATCATTCC
 1981 CCCCCAGACT CGTCTTACCT TTCCGGAGCG AACCTCAACC TCTCCTGCCA CTCGGCTCT
 2041 AACCCATCCC CGCACTTAC TTGGCGTATC AATGGGGATAC CGCAGCAACA CACACAAGTT
 2101 CTCTTATCG CAAAATCAC GCCAAATAAAT AACGGGACCT ATGCCTGTTT TGTCTAAC
 2161 TTGGCTACTG GCCGCAATAA TTCCATAGTC AAGAGCATCA CAGTCTCTGC ATCTGAACT
 2221 TCTCCTGGTC TCTCAGCTGG GGCCACTGTC GGCATCATGA TTGGAGTGCT GGTTGGGTT
 2281 GCTCTGATAT AGTTTTATC TCGAGGGATC CCCCCGGGTAG CTAGCTAATT TTTCTTTAC
 2341 GTATTATAT

FIG.23

SUBSTITUTE SHEET (RULE 26)

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1 ATGTACAGCATGCAGCTCGATCCTGTGTACATTGACACTTGTGCTCCTGTCAACAGC
 61 GCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCACAGCAGCAGCAGCAG
 121 CAGCAGCAGCAGCAGCAGCACCTGGAGCAGCTGTGATGGACCTACAGGAGCTCCTGAGC
 181 AGGATGGAGAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCTCAAATTACTTG
 241 CCCAAGCAGGCCACAGAATTGAAAGATCTCAGTGCCTAGAAGATGAACTTGGACCTCTG
 301 CGGCATGTTCTGGATTGACTCAAAGCAAAGCTTCAATTGGAAGATGCTGAGAATTTC
 361 ATCAGCAATATCAGAGTAACTGTTGAAACTAAAGGGCTGACAAACACATTGAGTGC
 421 CAATTGATGAGTCAGCAACTGTTGAGGAGATGGATAGCCTCTGT
 481 CAAAGCATCATCTCAACAAGCCCTCAATAA

FIG.24

1 ATGTACAGGATGCAACTCCTGTCTGCATTGCACTAAGTCTTGCACATTGTCACAAACAGT
 61 GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACACTGGAGCATTACTCTGGAT
 121 TTACAGATGATTTGAATGGAATTAATAATTACAAGAATCCAAACTCACCAGGATGTC
 181 ACATTTAAGTTTACATGCCAAGAAGGCCACAGAACTGAAACATCTCAGTGTCTAGAA
 241 GAAGAACTCAAACCTCTGGAGGAAGTGTCAAATTAGCTCAAAGCAAAACTTTCACTTA
 301 AGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACCTAAAGGGATCTGAA
 361 ACAACATTGATGTAATATGCTGATGAGACAGCAACCATTGAGAATTCTGAACAGA
 421 TGGATTACCTTTGTCAAAGCATCATCTCAACACTGACTTGA

FIG.25

1 ACATCATGCAGTGGTAAACAAAAACATTTTATTCTCAAATGAGATAAAGTAAAAATAT
 61 ATATCATTATATTACAAAGTACAATTATTTAGGTTAATCATGAAACCTACACACTGCAT
 121 CTTGGCTTGCAGCTCTCCTCATGGCTTTCTGGCTGTTACTGCCACGGCACAGTCAT
 181 TGAAAGCTAGAAAGTCTGAATAACTATTTAACTCAAGTGGCATAGATGTGGAAGAAAA
 241 GAGTCTCTTCTGGATATCTGGAGGAACCTGGCAAAAGGATGGTACATGAAAATCCTGCA
 301 GAGCCAGATTATCTCTTCTACCTCAGACTCTTGAAGTCTGAAAGACAATCAGGCCAT
 361 CAGCAACAACTAACGTCATTGAATCACACCTGATTACTACCTTCTTCAGCACAGCAA
 421 GGCAGAAAAGGATGATTGAGTATTGCAAGTTGAGGTCAACAAACCCACAGGTCCA
 481 GCGCCAAGCATTCAATGAGCTCATCCAGTGGTCCACCAGCTGTTGCCGGAATCCAGCCT
 541 CAGGAAGCGGAAAAGGAGTCGCTGCTGATTGGGGTGGGGAAAGAGATTGTCCTAA

FIG.26

1 ACATCATGCAGTGGTAAACAAAAACATTTTATTCTCAAATGAGATAAAGTAAAAATAT
 61 ATATCATTATATTACAAAGTACAATTATTTAGGTTAATCATGAAATATACAAGTTATAT
 121 CTTGGCTTTCAGCTCTGCATCGTTGGTTCTCTGGCTGTTACTGCCAGGACCCATA
 181 TGTAAGAAGCAGAAAACCTTAAGAAATATTTAATGCAGGTCAATTGAGATGTAGCGGA
 241 TAATGGAACCTTTCTTAGGCATTTGAAGAATTGAAAGAGGAGAGTACAGAAAAAT
 301 AATGCAGAGCAAATTGTCTCTTTACTTCAAACCTTTAAAAACTTAAAGATGACCA
 361 GAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAGTTTCAATAG
 421 CAACAAAAAGAAACGAGATGACTTCGAAAGCTGACTAATTTCGGTAACTGACTTGAA
 481 TGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGTGACTGCTGAGCTGAGCAGC
 541 TAAAACAGGGAGCGAAAAGGAGTCAGATGCTGTTCAAGGTCGAAGAGCATCCCAGTA
 601 A

FIG.27**SUBSTITUTE SHEET (RULE 26)**

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1 AAGCTTCTATCAAAGTCTTAATGAGTTAGGTAGATAGTATAGATATTACTACAAAGG
 61 TATTCAATTCTATCAATTCTAAAGTAGATGATATTAAACTCAAAGATGATGATAG
 121 TAGATAATAGATACGCTCATATAATGACTGCAAATTGGACGGTTCACATTTAATCATC
 181 ACGCGTTCATAGTTCAACTGCATAGATCAAATCTACTAAAAGATAGCCGATGTAT
 241 TTGAGAGAGATTGGACATCTAACTACGCTAAAGAAATTACAGTTATAAAATAACATAAT
 301 GGATTTGTTATCATCAGTTATTTAACATAAGTACAATAAAAGTATTAATAAAAT
 361 ACTTACTTACGAAAAATGTCATTATTACAAAACATATTACAGAACATCTATAGT
 421 AGAGTCCTTAAAGAGTTATAATTAAAAGATAACCATAATGTAATATTACACATCAGA
 481 TGATGATACTGTTAGTAATAAAATGAAGATAATGACTGTTATCTACAAGATTATTATC
 541 ATTTGATAAAATTCTGTTTTAATCCTTTAATAACGGTTATCAAATACGAAACTAT
 601 TAGTGATACAATATTAGATAGATACTCATATTATATACCTAGTTCTTCTTCTT
 661 GTTAGATATTCTAAAAAAAGAGCGTGTGATTTAGAATTAGAAGATCTAAATTATGCGTT
 721 AATAGGAGACAATAGTAACCTATATTATAAGATATGACTTACATGAATAATTGGTTATT
 781 TACTAAAGGATTATTAGATTACAAGTTGTATTATTGCGCGATGTAGATAATGTTACAA
 841 ACAGTATAATAAAAGAATACTATAATAGATATAACATCGCGATAACAGACAGTATAA
 901 CATATGGTTAAAATGTTAGAATACTGTTCTCTGGCTATATATTATGGTTACATGA
 961 TCTAAAGCCGCTGCTGAAGATGATTGGTTAGATACGATAACCGTATAACGAATTATC
 1021 TCGGGATAAAATTATACACTTCGAGTTCATAGTTATTAGAAAATAATATAAAACATT
 1081 ACGAGTAGGTACAATAATTGTACATCCAAACAGATAATAGCTAATGGTACATCTAATAA
 1141 TATACTTACTGATTCTCATCTACGTAGAAGAACTAATATCATCATAATTCTATCTAT
 1201 AATATTGGCCGGATATTTTAAAGATTCTTGAGGACCACTATTTCAGAATTATTTC
 1261 TTCACTCTCTGAATGGGTAAAGAATAGTAACGTTAGTACACCTGAAAACAGGGTATGA
 1321 AGCTATACTCTTGATGCTAGTTATTTCACACTCTACTAAAAGCAATTATGTAAA
 1381 ATATTGGACAAAGAAAACTTGCACTATAAGAACTTTAAAGACGGTAAACAGTTAGC
 1441 AAAATATATAATTAAAGAAAGATAGTCAGGTGATAGATAGAGTATGTTATTACACGCAGC
 1501 TGTATATAATCAGTAACCTTACTTAATGGATACGTTAAAATTCTGGTTGATTTAA
 1561 ATTCTCCGGAATGATAGATATACTACTGTTGGAAATTGCATAAGGATAATGAGAATAT
 1621 ATTATTCGAAACGTTCTGTAACATAATATAATCAGAATCTATCTATGCAAGATT
 1681 TTACTTATATCAGATGTTAAATTCACTGTTAAAGAATATAAAACTATGTTCC
 1741 TATACTCGCAGAAAACACTATCCAAAAGGAAGGCCCTATTTCACACATACATCTAACGA
 1801 AGATCTTCTGTCTATCTGTTATCGGAAGTAACAGTTGTAAGATATAAAATCCATT
 1861 ATTATATTCTAAAAGGATATATCAGCAAAACGATTCACTAGGTTATTACATCTGTCGA
 1921 TATAAATACGGCTGTTGAGTTAAGAGGATATAAAAGAGTAATAGGATGTTAGAATG
 1981 GCCTGAAAAGATAAAAATTTAATTCTAATCCTACATACATTAGATTACTAACAGA
 2041 AAGACGTTAGATATTCTACATTCTATCTGCTTAAATTAAATAACAGAGGATATAGC
 2101 TACCAGAGATGGAGTCAGAAATAATTACCTATAATTCTTTATCGTCAGTTATTGAG
 2161 ATCGTATACTTAAATTACTAAATTGCCATATGTCACATTCTGTAAGATAACAAAGTG
 2221 TAAATATAATCAGGTAATATATAATTCTATATAGGAGTATATAATTGAAAAGTAAA
 2281 TATAAATCATATAATAATGAAACGAAATATCAGTAATAGACAGGAACTGGCAGATTCTC
 2341 TTCTAATGAAAGTAACTGCTAAATCTCAAAATTAGATAAAAATGATAACAGCAAATAC
 2401 AGCTTCATTCAACGAATTACCTTTAATTTCAGACACACCTTATACAAACTAACTA
 2461 AGTCAGATGATGAGAAAAGTAAATATAAAATTAACTTATGGGTATAATATAAAAGATT
 2521 ATGATATTAAATTACTTAACGATGTTAATAGACTTATTCCATCAACCCCTCAAACC
 2581 TTTCTGGATATTATAAAATACCAGTTAATGATATTAAAATAGATTGTTAAGAGATGAA
 2641 ATAATTATTGGAGGTAAGGATATAAAATTAGTCTATCTTCACATGGAAATGAATTAC
 2701 CTAATATTAAATTATGATAGGAATTTCAGGATTACAGCTGTTATATGTATCAACA
 2761 ATACAGGGCAGATCTATGGTTATGGTAAACACTGTAACGGGAAGCAGCATTCTATGGTAA
 2821 CTGGCCTATGTTAATAGCCAGATCATTACTCTATAACATTTCACCACAAATAATAG
 2881 GATCCTCTAGATATTAAATTATCTAACACAAACAAAAAAATTAAACGATGATGGC
 2941 CAGAAGTATTCTACTAATAAAAGATAAAAGATAGTCTATCTTATCTACAAAGATATGAAAG
 3001 AAGATAATCATTAGTAGCTACTAATATGGAAAGAAATGTATACAAAACGTGGAAG
 3061 CTT

FIG.28

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1 GAGCTCGGGCCGCTATCAAAAGTCTTAATGAGTTAGGTAGATAGTATAGATATTAC
 61 TACAAAGGTATTCATATTCCTATCAATTCTAAAGTAGATGATATTAAACTCAAAGAT
 121 GATGATAGTAGATAATAGATACGCTCATATAATGACTGCAAATTGGACGGTCACATT
 181 TAATCATCACGCGTCAAGTTCAACTGCATAGATCAAATCTCACTAAAAGATAGC
 241 CGATGTATTGAGAGAGATTGGACATCTAACTACGCTAAAGAAATTACAGTTATAAATAA
 301 TACATAATGGATTGTTATCATCAGTTATTTAACATAAGTACAATAAAAGTATTAA
 361 ATAAAAAATACTTACTTACGAAAATGACTAATTAGCTATAAAACCCGGCTGCAGCTCG
 421 AGGAATTCTTTTATTGATTAACAGTCAAATGAGTATATATAATTGAAAAGTAAAATA
 481 TAAATCATATAATAATGAAACGAAATATCAGTAATAGACAGGAACCTGGCAGATTCTTCTT
 541 CTAATGAAGTAAAGTACTGCTAAATCTCAAAATTAGATAAAAATGATAACAGCAAACACAG
 601 CTTCATTCACGAAATTACCTTTAATTTCAGACACACCTTATTACAAACTAACTAAG
 661 TCAGATGATGAGAAAGTAAATATAAATTAACTTATGGGTATAATATAATAAGATTCAT
 721 GATATTAAATAATTACTAACGATGTTAATAGACTTATTCCATCAACCCCTTCAAACCTT
 781 TCTGGATATTATAAAATACCAAGTTAATGATATTAAATAGATTGTTAAGAGATGTAAT
 841 AATTATTGGAGGTAAAGGATAAAAATTAGTCTATCTTCACATGGAAATGAATTACCT
 901 AATATTAAATAATTATGATAGGAATTTCAGGATTACAGCTGTTATGTATCAACAAAT
 961 ACAGGCAGATCTATGGTTATGGTAAACACTGTAACGGGAACGAGCAGCATTCTATGTAAC
 1021 GGCCTATGTTAATAGCCAGATCATTACTCTATAAACATTTCACACAAATAATAGGA
 1081 TCCTCTAGATATTAAATTATCTAACACACAAAAATTAAACGATGTATGGCCA
 1141 GAAGTATTTCTACTAATAAAGATAAAGATAGTCTATCTTATCTACAAGATATGAAAGAA
 1201 GATAATCATTAGTAGCTACTAATATGAAAGAAATGTATACAAAACGTGAAAGCT
 1261 TTTATATTAAATAGCATATTACTAGAAGATTAAATCTAGACTTAGTATAACAAACAG
 1321 TTAAATGCCAATATCGATTCTATATTTCATCATAACAGTAGTACATTAACTCAGTGTATA
 1381 CTGAAACGATCTACAGACTCAACTATGCAAGGAATAAGCAATATGCCAATTATGCTAAT
 1441 ATTTAACCTTTAGAACTAAACGTTCTACCAATACTAAAATAGGATACGTGATAGGCTG
 1501 TTAAAAGCTGCAATAAATAGTAAGGATGTTAGAAGAAATACCTTGTATACCTCGGAG
 1561 GAAAGAACTTTAGAACAACTTAAGTTAATCAAACCTTGTATTTATGAAGGTACC

FIG.29

1 GAATTCGAATAAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTGTTAGTTCTC
 61 TCTAAAATGGGCTCAACCCCCAGCTAGTTGTCATCCTGCTTTCTCGAATGTAC
 121 CAGGAGCCATATCCACGGATGCGACAAAATCACTTGAGAGAGATCATGGCATTGAA
 181 CGAGGTACAGGAGAAGGGACGCCATGCACGGAGATGGATGTGCCAACGTCTCACAGC
 241 AACGAAGAACACACAGAGAGTGGCTCTGTAGGGCTCCAAGGTGCTTCGTATATT
 301 TTATTTAAACATGGAAAACCTCCATGCTTGAAGAAGAACTCTAGTGTCTCATGGAGCT
 361 CGAGAGACTCTTCGGGCTTTGATGCCGGATTATCGATAAGCTGCACCATGAAATGGA
 421 GTCCAAGTCCACATCACTGAAAGACTCCTGGAAAGCCTAAAGAGCATCATGCAAATGGA
 481 TTACTCGTAG

FIG.30

1 GAATAAAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTGTTAGTTCTCTCTAAA
 61 AATGGGCTCACCTCCCAACTGCTTCCCCCTCTGTTCTCTGCTAGCATGTGCCGCAA
 121 CTTTGTCCACGGACACAAGTGCATATCACCTAACAGGAGATCATAAAACATTGAACAG
 181 CCTCACAGAGCAGAAGAGACTCTGTGCACCGAGTTGACCGTAACAGACATCTTGCTGCCTC
 241 CAAGAACACAACGTGAGAAGGAAACCTCTGCAGGGCTGCGACTGTGCTCCGGCAGTTCTA
 301 CAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCA
 361 CAAGCAGCTGATCCGATTCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGGGCTT
 421 GAATTCTGTCTGTGAAGGAAGCCAACCAAGAGTACGTTGGAAAACCTTCTGGAAAGGCT
 481 AAAGACGATCATGAGAGAGAAATTCAAAGTGTGAGCTGA

FIG.31**SUBSTITUTE SHEET (RULE 26)**

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1 GAATAAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTGTTAGTTCTCTCTAA
 61 AATGTGGCTGCAGAGCCTGCTCTGGCACTGTGGCTGCAGCATCTCTGCACCCGC
 121 CCGCTGCCAGCCCCAGCACGCAGCCCTGGGAGCATGTGAATGCCATCCAGGAGGCCG
 181 GCGTCTCTGAACCTGAGTAGAGACACTGCTGAGATGAATGAAACAGTAGAAAGTCAT
 241 CTCAGAAATGTTGACCTCCAGGAGCCACCTGCCTACAGACCCGCTGGAGCTGTACAA
 301 GCAGGGCTGCGGGCAGCCTCACCAAGCTCAAGGGCCCTGACCATGATGCCAGCCA
 361 CTACAAGCAGCACTGCCCTCCAACCCGGAAACTTCCGTGCAACCCAGACTATCACCTT
 421 TGAAAGTTCAAAGAGAACCTGAAGGACTTCTGCTGTACCCCCTTGACTGCTGGGA
 481 GCCAGTCAGGAGTGA

FIG.32

1 CAAAATTGAAAATATATAATTACAATATAAAATGTGTCAACCAGCAGTTGGTCATCTCTTG
 61 GTTTCCCTGGTTTCTGGCATCTCCCCTCGTGGCCATATGGAACTGAAGAAAGATGT
 121 TTATGTCGTTAGAATTGGATTGGATTCGGATGCCCTGGAGAAATGGGGTCTCACCTG
 181 TGACACCCCTGAAGAAGATGGTATCACCTGGACCTGGACAGAGCAGTGAGGTCTTAGG
 241 CTCTGGCAAAACCCCTGACCATCCAAGTCAAAGAGTTGGAGATGCTGCCAGTACACCTG
 301 TCACAAAGGAGGCAGGGTTCTAACGCCATTGCTCTGCTGCTCACAAAAGGAAGATGG
 361 AATTTGGTCCACTGATATTAAAGGACCAAGAAAGAACCCAAAATAAGACCTTCTAAG
 421 ATGCGAGGCCAAGAATTATTCTGGACGTTCACCTGCTGGGGCTGACGACAATCAGTAC
 481 TGATTTGACATTCACTGTCAAAAGCAGCAGAGGCTTCTGACCCCCAAGGGGTGACGTG
 541 CGGAGCTGCTACACTCTGCAAGAGAGTCAGAGGGACAACAAGGAGTATGAGTACTC
 601 AGTGGAGTGCCAGGAGGACAGTGCCTGCCAGCTGCTGAGGAGAGTCTGCCATTGAGGT
 661 CATGGTGGATGCCGTTCACAGCTCAAGTATGAAAACACTACACCAGCAGCTTCTCATCAG
 721 GGACATCATCAAACCTGACCCACCCAGAAACTTGCAAGCTGAAGCCATTAAAGAATTCTG
 781 GCAGGTGGAGGTCAAGCTGGAGTACCCCTGACACCTGGAGTACTCCACATTCTACTTCTC
 841 CCTGACATTCTGCGTTCAAGTCCAGGGAAGAGCAAGAGAGAAAAGAAAGATAGAGTCTT
 901 CACGGACAAGACCTCAGCCACGGTCACTGCGCAAAATGCCAGCATTAGCGTGCAGG
 961 CCAGGACCGCTACTATAGCTCATCTGGAGCGAATGGCATTGTGCCCTGCAAGTTAG

FIG.33

1 GAATAAAAAATGATAAAGTAGGTTCAGTTTATTGCTGGTTGTGTTAGTTCTCTCTAA
 61 AATGTGTCAGCGCCAGCCTCCTCTGGCTACCCCTGGCTCCTGGACCACCTCAG
 121 TTTGGCCAGAAACCTCCCCGTGGCACTCCAGACCCAGGAATGTTCCCATGCCCTCACCA
 181 CTCCCAAAACCTGCTGAGGGCCGTCAAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGA
 241 ATTTTACCCCTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACAG
 301 CACAGTGGAGGCCTGTTTACCAATTGAAATTAAACCAAGAATGAGAGTTGCCCTAAATTCCAG
 361 AGAGACCTCTTCAACTAAATGGAGTTGCCTGCCCTCCAGAAAGACCTCTTTATGAT
 421 GGCCCTGTGCCCTAGTAGTATTATGAAGAGACTTGAGATGATCAGGTTGGAGTTCAAGAC
 481 CATGAATGCAAAGCTCTGATGGATCCTAAGAGGCAGATCTTCTAGATCAAACATGCT
 541 GGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTCAACAGTGAAGACTGTGCCACAAA
 601 ATCCTCCCTGAAGAACGGATTTTATAAAACTAAAATCAAGCTCTGCATACTTCTCA
 661 TGCTTCAGAATTGGCAGTGAATTGACAGAGTGAAGCTATCTGAATGCTTCCTA
 721 A

FIG.34

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1 ATGGCTTGCATTGTCAGTTGATGCAGGATAACCACTCCTCAAGTTCCATGTCCAAGG
61 CTCATTCTCTCTTGTGCTGCTGATTGCTTACAAGTGTCTTCAGATGTTGATGAA
121 CAACTGTCCAAGTCAGTGAAAGATAAGGTATTGCTGCCTTGCCTTACAACACTCCTCAT
181 GAAGATGAGTCTGAAGACCGAATCTACTGGCAAAACATGACAAAGTGGTGTGCTGTC
241 ATTGCTGGAAACTAAAAGTGTGGCCCGAGTATAAGAACCGGACTTATATGACAACACT
301 ACCTACTCTTATCATCCTGGCCTGGCTTCAAGACCGGGCACATACAGCTGTGTC
361 GTTCAAAAGAAGGAAAGAGGAACGTATGAAGTTAACACTTGGCTTAGTAAAGTTGTCC
421 ATCAAAGCTGACTTCTCTACCCCCAACATAACTGAGTCTGGAAACCCATCTGCAGACACT
481 AAAAGGATTACCTGCTTGCTTCCGGGGTTCCCAAAGCCTCGCTTCTCTGGTTGGAA
541 AATGGAAGAGAATTACCTGGCATCAATACGACAATTCCAGGATCCTGAATCTGAATTG
601 TACACCATTAGTAGCCAACTAGATTCATAACGACTCGCAACCACACCATTAAAGTGTCTC
661 ATTAAATATGGAGATGCTCACGTGTCAGAGGACTTCACCTGGAAAAACCCCCAGAAGAC
721 CCTCCTGATAGCAAGAACACACTTGTGCTCTTGGGCAGGATTGGCGCAGTAATAACA
801 GTCGTCGTATCGTTGTCATCATCAAATGCTTCTGTAAGCACAGAAGCTTTCAAGAAGA
861 AATGAGGCAAGCAGAGAAACAAACAGCCTTACCTTGGCCTGAAGAAGCATTAGCT
901 GAACAGACCGTCTCCTTTAG

FIG.35

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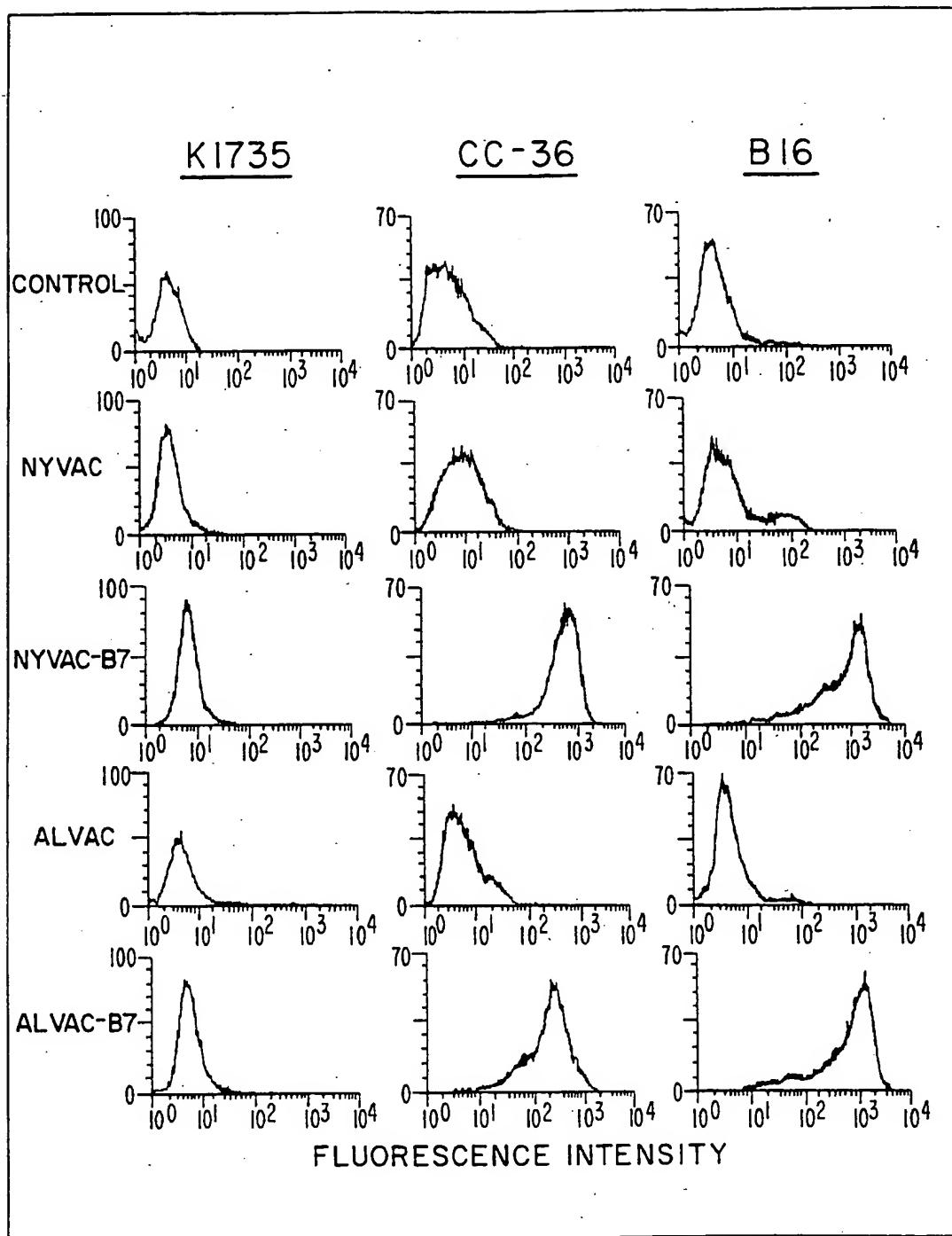


FIG.36

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1 ATGGGCCACACACGGAGGCAGGGAACATCACCATCCAAGTGTCCATAACCTCAATTCTTT
 61 CAGCTCTGGTGTGGCTGGCTTTCTCACTTCTGTTCAAGGTGTTATCCACGTGACCAAG
 121 GAAGTGAAGAAGTGGCAACGCTGTCTGTGGTACAATGTTCTGTTGAAGAGCTGGCA
 181 CAAACTCGCATCTACTGGCAAAAGGAGAAGAAAATGGTGTGACTATGATGTCTGGGAC
 241 ATGAATATATGGCCCGAGTACAAGAACCGGACCATCTTGATATCACTAATAACCTCTCC
 301 ATTGTGATCCTGGCTCTGCGCCCACATCTGACGAGGGCACATACGAGTGTGTTCTGAAG
 361 TATGAAAAGACGCTTCAAGCGGGAACACCTGGCTGAAGTGACGTTATCAGTCAAAGCT
 421 GACTTCCCTACACCTAGTATACTGACTTTGAAATTCCAACCTCTAATATTAGAAGGATA
 481 ATTTGCTCAACCTCTGGAGGTTTCCAGAGCCTCACCTCTCCTGGTTGAAAATGGAGAA
 541 GAATTAATGCCATCAACACAACAGTTCCAAGATCCTGAAACTGAGCTCTATGCTGTT
 601 AGCAGCAAACCTGGATTTCATGACAACCAACCACAGCTTCATGTTCTCATCAAGTAT
 661 GGACATTTAAGAGTGAATCAGACCTCAACTGGAATACAACCAAGCAAGAGCATTTCCT
 721 GATAACCTGCTCCCATCTGGCCATTACCTTAATCTCAGTAAATGGAATTTTGATA
 781 TGCTGCCCTGACCTACTGCTTGGCCCAAGATGCAGAGAGAGAAGGAGGAATGAGAGATTG
 841 AGAAGGGAAAGTGTACGCCCTGTATAA

FIG.37

1 ATGACTGCCATGGAGGAGTCACAGTCGGATATCAGCCTCGAGCTCCCTGAGCCAGGAG
 61 ACATTTCAAGCTTATGGAACACTACTTCCCTCCAGAAGATATCCTGCCATCACCTCACTGC
 121 ATGGACGATCTGTTGCTGCCCAAGGATGTTGAGGAGTTTTGAAGGCCAAGTGAAGCC
 181 CTCCGAGTGTCAAGGAGCTCCTGCAGCACAGGACCCCTGTCACCAGAGACCCCTGGCCAGTG
 241 GCCCCCTGCCCAAGCCACTCCATGGCCCCCTGTCATCTTGTCCCTCTCAAAAAACTTAC
 301 CAGGGCAACTATGGCTTCCACCTGGGCTTCCCTGCAGTCTGGGACAGCCAAGTCTGTTATG
 361 TGCACGTACTCTCCCTCCCTCAATAAGCTATTCTGCCAGCTGGCGAAGACGTGCCCTGTG
 421 CAGTTGTGGGTCAAGGCCACACCTCCAGCTGGGAGCCGTGCCCGGCCATGGCCATCTAC
 481 AAGAAGTCACAGCACATGACGGAGGTGAGACGCTGCCCAACCATGAGCGCTGCTCC
 541 GATGGTGATGGCTGGCTCCCTCCCCAGCATCTTATCCGGTGGAAAGGAAATTGTATCCC
 601 GAGTATCTGGAAGACAGGCAGACTTTCGCCACAGCGTGGTGGTACCTTATGAGCCACCC
 661 GAGGCCGGCTCTGAGTATACCAACCATCCACTACAAGTACATGTTAATAGCTCCTGCATG
 721 GGGGCATGAACGCCGACCTATCCTTACCATCATCACACTGGAAGACTCCAGTGGGAAC
 781 CTTCTGGGACGGGACAGCTTGAGGTTGCTGTTGTGCCTGCCCTGGAGAGACCGCCGT
 841 ACAGAAGAAGAAAATTCCGCAAAAGGAAAGTCCCTTGCCTGAACTGCCCAAGGGAGC
 901 GCAAAGAGAGCGCTGCCACCTGCACAAGCGCCTCTCCCCGCAAAAGAAAAACCACTT
 961 GATGGAGAGTATTCACCCCTAAGATCCGGGGCGTAAACGCTTCGAGATGTTCCGGGAG
 1021 CTGAATGAGGCCTTAGAGTAAAGGATGCCATGCTACAGAGGAGTCTGGAGACAGCAGG
 1081 GCTCACTCCAGCTACCTGAAGACCAAGAAGGGCAGTCTACTTCCGCCATAAAAAAAC
 1141 ATGGTCAAGAAAGTGGGGCTGACTCAGACTGA

FIG.38

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1 ATGGAGGAGCCGCACTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTCA
61 GACCTATGGAAACTACTTCCTGAAAACAACGTTCTGTCCCCCTGCCGTCAGCAATG
121 GATGATTGATGCTGTCGGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCA
181 GATGAAGCTCCCAGAATGCCAGAGGCTGCTCCCCCGTGGCCCTGCACCAGCAGCTCCT
241 ACACCGGGCGGCCCTGCACCAGCCCCCTCCTGGCCCTGTCATCTTCTGTCCCTTCCCAG
301 AAAACCTACCAGGGCAGCTACGGTTCCGTCTGGGCTTCTTGCATTCTGGACAGCCAAG
361 TCTGTGACTTGACCGTACTCCCCTGCCCTCAACAAGATGTTTGCCAATGGCCAAGACC
421 TGCCCTGTGCAGCTGTGGTTGATTCCACACCCCCGCCGCACCCGCGTCCGCGCCATG
481 GCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCCTGCCCTCACCACATGAG
541 CGCTGCTCAGATAGCGATGGTCTGGCCCTCCTCAGCATCTTATCCGAGTGGAAAGGAAAT
601 TTGCGTGTGGAGTATTGGATGACAGAAAACACTTTTGACATAGTGTGGTGGTGCCTAT
661 GAGCCGCCTGAGGTTGCCTTGACTGTACCAACATACAACATGTGTAACAGT
721 TCCGTGATGGCGGCATGAACCGGAGGCCATCCCTACCATCATCACACTGGAAGACTCC
781 AGTGGTAATCTACTGGGACGGAACAGCTTGAGGTGCGTGTGCTGTCTGGGAGA
841 GACCGGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAAGGGGAGCCTCACACGAGCTGCC
901 CCAGGGAGCACTAAGCGAGCACTGCCAACACACCAACAGCTCTCTCCCCAGCCAAAGAAG
961 AAACCACGGATGGAGAATATTCACCCCTCAGATCCGTGGCGTGAGCGCTCGAGATG
1021 TTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGGATGCCAGGCTGGGAAGGAGCCAGGG
1081 GGGAGCAGGGCTCACTCCAGCCACCTGAAGTCCAAAAGGGTCAGTCTACCTCCGCCAT
1141 AAAAAACTCATGTCAAGACAGAAGGGCTGACTCAGACTGA

FIG.39

INTERNATIONAL SEARCH REPORT

International application No.
PC/US94/00888

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :424/88 93A, 93T; 435/69.3, 69.5, 172.3, 320.1; 530/350, 828

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88 93A, 93T; 435/69.3, 69.5, 172.3, 320.1; 530/350, 828

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, DERWENT, EMBASE

search terms: vaccinia, tumor, cytokine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Virology, Volume 188, Number 1, issued May 1992, J. Tartaglia et al., "NYVAC: A Highly Attenuated Strain of Vaccinia Virus", pages 217-232, see entire document.	1-13, 19-22
Y	Virology, Volume 190, Number 1, issued September 1992, E. Konishi et al., "A Highly Attenuated Host Range-Restricted Vaccinia Virus Strain, NYVAC, Encoding the prM, E, and NS1 Genes of Japanese Encephalitis Virus Prevents JEV Viremia in Swine", pages 454-458, see entire document.	1-13, 19-22
Y	The Lancet, Volume 339, Number 8807, issued 13 June 1992, M. Cadoz et al., "Immunisation with Canarypox Virus Expressing Rabies Glycoprotein", pages 1429-1432, see entire document.	1-2, 15-22

 Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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• "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 APRIL 1994

Date of mailing of the international search report

04 MAY 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00888

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Cellular Biochemistry, Supplement 16B, 25 January 1992, K.L. Elkins et al., "In Vivo Delivery of Interleukin 4 and Prevention of Tumor Development Using a Recombinant Vaccinia Virus", page 282, see entire abstract.	1-3, 20-22
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Y		4-19
X	Journal of Cellular Biochemistry, Supplement 15F, 01 April 1991, I.A. Ramshaw et al., "The Analysis of Immune Effector Mechanisms Using Recombinant Vaccinia Viruses that Express their own Cytokines", page 127, see entire abstract.	1-3, 20-22
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Y		4-19
X	Immunological Reviews, No. 127, issued June 1992, I. Ramshaw et al., "Expression of Cytokines by Recombinant Vaccinia Viruses: A Model for Studying Cytokines in Virus Infections In Vivo", pages 157-182, see entire document.	1-3, 20-22
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Y		4-19
X	Nucleic Acids Research, Volume 20, Number 3, issued 11 July 1992, D. Ronen et al., "Expression of Wild-Type and Mutant p53 Proteins by Recombinant Vaccinia Viruses", pages 3435-3441, see entire document.	1-3, 20-22
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Y		4-19
X	Cancer Research, Volume 52, issued 15 December 1992, J. Kantor et al., "Immunogenicity and Safety of a Recombinant Vaccinia Virus Vaccine Expressing the Carcinoembryonic Antigen Gene in a Nonhuman Primate", pages 6917-6925, see entire document.	1-3, 20-22
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Y		4-19
X	Proceedings of the National Academy of Sciences USA, Volume 85, issued February 1988, C.D. Estin et al., "Recombinant Vaccinia Virus Vaccine Against the Human Melanoma Antigen p97 for use in Immunotherapy", pages 1052-1056, see entire document.	1-3, 20-22
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Y		4-19
X	Proceedings of the National Academy of Sciences USA, Volume 88, issued May 1991, S.K. Sambhi et al., "Local Production of Tumor Necrosis Factor Encoded by Recombinant Vaccinia Virus is Effective in Controlling Viral Replication In Vivo", pages 4025-4029, see entire document.	1-3, 20-22
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Y		4-19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/00888

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 37/00, 37/66, 39/295; C07K 15/00, 15/26; C12N 7/00, 7/01, 15/19, 15/63, 15/86

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